# PROCEEDINGS

OF THE

# THIRD INTERNATIONAL CONGRESS

INTL. CONG.

OF

# BIOCHEMISTRY

BRUSSELS 1955

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ACADEMIC PRESS INC., PUBLISHERS — NEW YORK, N. Y
1956

**Reference**

*Proceedings of the third international congress of biochemistry, Brussels 1955* (C. Liébecq, edit
Academic Press, New York 1956.

Printed in Belgium

# FOREWORD

The Organising Committee and the Scientific Committee of the 3rd International Congress of Biochemistry are proud and happy to be able now to publish the text of the lectures, reviews and discussions of reviews which were presented at the Congress.

The Scientific Committee under the guidance of its original President, Professor Marcel Florkin, made every effort to give in the programme of the Congress as complete a picture as possible of the present position of Biochemistry in the world as a whole : it was in fact especially important to show that biochemistry is very much alive and undergoing development on a wide front at a time when, as a science, it had at last achieved official recognition as an independent discipline through the creation of the International Union of Biochemistry.

It is for this reason that, apart from sessions for members own communications, the Congress also included reviews followed by discussions and also set lectures. The lectures, reviews and discussions were entrusted to eminent Specialists drawn from all countries. Those who had the opportunity to be present at these sessions were able to appreciate the interest of such surveys and the liveliness of the discussions which followed them.

The Scientific Committee of the Congress in publishing the text of the lectures, reviews and discussions in the present volume hopes thereby to make a contribution to the progress of Biochemistry ; it would have been most regrettable if the expositions and surveys made by so many distinguished biochemists were lost to the general reader through lack of publication.

It is proper to recall that the text of the reviews was sent before the Congress to the persons who had been invited by the Directors of the Sections to participate in the discussion of those reviews. At the time of the Congress proofs of the reviews were distributed to all members ; this method of preparing for the discussion of reviews seems to have produced very good results.

The Scientific Committee of the Congress has only been able to publish contributions to the discussions in cases where the script reached the Committee in time ; the length of such scripts has had to be strictly limited for reasons of economy.

The present publication is actually very costly and its appearance has only been made possible thanks to the pecuniary support which the President of the Finance Committee, Professor Paul Putzeys, has succeeded in enlisting. The Scientific Committee of the 3rd International Congress of Biochemistry wishes to express its deepest thanks and gratitude to all those who have helped to make this publication possible : — first, the authors of the lectures, reviews and discussions who have given their time and trouble in preparing the articles presented ; next, the organisations and private persons who have given us financial help, the Directors of Sections, Professors Z. M. Bacq, L. Brull, H. Chantrenne, M. J. Dallemagne, C. de Duve, V. Desreux, M. Dubuisson, P. E. Grégoire, H. Koch, P. Manil, R.Martin, L. Massart, P. Putzeys, P. Simonart, L. Vandendriessche and M. Welsch who have spared no effort to ensure the scientific success of the Congress ; finally to the Secretary-General of the Congress, Professor Claude Liébecq, and to the scientific secretaries Madame Liébecq-Hutter and Dr. W. G. Verly.

Without the unremitting labours of these people the publication of the present volume would have been impossible.

J. BRACHET.

President of the Scientific Committee.

# CONTENTS

# Thyroxin und oxydative Phosphorylierung (*)

von C. MARTIUS

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(*Eingegangen am* 11. *Juli* 1955)

*Die Wirkung des Schilddrüsenhormones* in vivo

Die Frage, wie die Wirkstoffe des pflanzlichen und tierischen Organismus d.h. die Vitamine und Hormone ihre spezifische Wirkung in der Zelle entfalten, gehört zu den interessantesten und wichtigsten Problemen der Biochemie.

Während man über den Wirkungsmechanismus der meisten wasserlöslichen Vitamine relativ schnell nach ihrer Auffindung und chemischen Bearbeitung Aufschluss erhalten konnte, haben die fettlöslichen Vitamine und vor allen die ganze Gruppe der Hormone entsprechenden Bemühungen hinter das Geheimnis ihrer Wirkung zu kommen bisher erfolgreich Widerstand geleistet. Man wird heute annehmen dürfen, dass all die genannten Stoffe letzten Endes ihre Wirkung durch Beeinflussung enzymatischer Vorgänge ausüben sei es, dass sie selbst Bestandteile von solchen sind, wie die Vitamine, oder (wie vermutlich die Hormone) in enzymatische Vorgänge steuernd eingreifen. Letzteres ist allerdings zur Zeit noch weitgehend Hypothese, denn selbst im Falle des Insulins, über dessen Wirkungsmechanismus wir relativ noch am besten Bescheid wissen, sind die letzten Einzelheiten der Wirkung bekanntlich noch ungeklärt.

Im Falle des Hormones der Schilddrüse haben Untersuchungen der letzten Jahre zu Ergebnissen geführt, die es gestatten, den Ort seines Eingreifens in die Stoffwechselvorgänge genau anzugeben und auch bereits Aussagen über den molekularen Mechanismus dieser Reaktion zu machen. Im Verlauf dieser Untersuchungen haben sich dann ganz überraschend Einblicke in den Wirkungsmechanismus zweier fettlöslicher Vitamine ergeben.

Da Störungen der hormonalen Steuerung durch die Schilddrüse zu den verbreitetsten und sozial wichtigsten endokrinen Erkrankungen gehören, liegt seit langem ein überaus reichhaltiges Beobachtungsmaterial klinischer und allgemein biologischer Art über das Schilddrüsenhormon vor. Wenn die Disfunktion dieses Organes auch zu sehr mannigfaltigen Erscheinungen am Gesamt-Organismus führt und kaum ein Organ verschont, steht die Wirkung auf den Basalstoffwechsel doch so eindeutig im Vordergrund, dass ernsthafte Zweifel daran, dass hier der Angriffspunkt des Hormones zu suchen sei, sich niemals behaupten konnten. Dass diese Wirkung humoraler Art sei und an jeder Zelle angreife, steht ebenfalls seit langem fest und wird höchstens noch von klinischer Seite manchmal bezweifelt. Dass man sich indessen über die Art des Eingreifens so lange noch nicht einmal eine angenäherte Vorstellung oder Arbeitshypothese machen konnte lag daran, dass das System der Energieproduktion, welches im Organismus Angriffspunkt des Hormones bildet, in seinem Aufbau und seinen Funktionen erst in der letzten Zeit näher erforscht wurde. Damit ist es vielleicht überhaupt erst Gegenstand allgemeineren Interesses bei den Biochemikern geworden.

Von grosser Wichtigkeit für das Problem der Beeinflussung des Energiestoffwechsels ist die Beobachtung geworden (1, 2), dass sich in dem Process der Atmungskettenphosphorylierung die eigentlichen Verbrennungsvorgänge (letzten Endes also die Knallgasreaktion $H^2 + O = H^2O$) von den Phosphorylierungsvorgängen, d. h. im Endeffekt der Reaktion

Adenosindiphosphat + Phosphat + 9 Kal. $\rightleftarrows$ Adenosintriphosphat

trennen lässt (« Entkopplung » von Atmung und Phosphorylierung). Hierdurch war auf eine Möglichkeit hingewiesen, die kalorigene Wirkung des Schilddrüsenhormones kausal zu verstehen. Unabhängig davon, ob man sich eine solche Entkopplung in der Weise vorstellt, dass durch hydrolytische Spaltungsvorgänge — etwa die Wirkung von ATP spaltenden Enzymen — Energie in Form energiereicher Bindungen direkt vernichtet d. h. in Wärme verwandelt wird, oder ob man einen anderen Mechanismus hierfür annimmt, wie im folgenden versucht werden soll zu zeigen, stellt die Annahme einer derartigen Beeinflussung des Energiestoffwechsels wohl die einzige Möglichkeit dar, sich das Wesen der Hormonwirkung plausibel zu machen.

Nachdem mehrere Versuche, eine solche Wirkung des Thyroxins auf die oxydative Phosphorylierung nachzuweisen, zunächst negative Resultate ergeben hatten (3, 4), ist das Problem jetzt von mehreren Seiten mit ubereinstimmend positiven Ergebnissen bearbeitet worden (5-10). Ueber derartige Untersuchungen soll im folgenden zusammenfassend referiert werden.

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(*) Vortrag gehalten auf der Eröffnungssitzung am 1. August 1955.

Unsere vor 5 Jahren begonnenen Versuche haben zunächst ergeben, dass die Zufuhr genügender Mengen von Thyroxin bei Ratten zu einer verminderten oxydativen Phosphorylierung in den isolierten Lebermitochondrien führt. Zur Messung der Phosphorylierungsrate wandten wir anfangs die von Lehninger (11) angegebene Methode des Einbaues von radioaktivem Phosphat in ATP an, später verwendeten wir ausschliesslich die Methode der Bestimmung der Messung des P : O Quotienten unter Verwendung von Hexokinase und Glukose als Phosphatakzeptor. Diese Versuche stiessen zunächst auf scharfe Kritik, da ihre Ergebnisse entweder nicht reproduziert werden konnten, oder die Höhe der von uns verwendeten Thyroxindosen es zweifelhaft erschienen liess, ob die beobachteten Effekte noch als physiologisch anzusehen seien. Auf Grund einer grossen Zahl von inzwischen durchgeführten Versuchen müssen wir den Einwand der Nichtreproduzierbarkeit unserer Versuche zurückweisen. Zur Entkräftigung des zweiten Einwandes wurden Versuchsreihen unternommen, in denen wir gleichzeitig die Wirkung des Schilddrüsenhormones auf die oxydative Phosphorylierung und den Grundumsatz bestimmten (6, 13). Es wurde hierbei bei zwei möglichst gleichen Versuchstieren, Meerschweinchen und Ratten, zunächst der Grundumsatz bestimmt, dann erhielt ein Tier im Laufe mehrerer Tage Thyroxin, bis eine ausreichende Erhöhung des Grundumsatzes eingetreten war (20-80 %). Dann wurden beide Tiere getötet und der P : O Quotient in den Lebermitochondrien und dem zu kleinen Stücken zerschnittenen Zwerchfell (12) bestimmt. Das Kontrolltier dient in diesem Versuch lediglich dazu, um den Abfall der Phosphorylierungsrate zu messen, da besonders bei Lebermitochondrien die gemessenen P : O Quotienten ziemlich empfindlich sind gegen Änderungen der Versuchsanordnung wie Aktivität des Hexokinasepräparates, Dauer der Präparation usw., die nicht in allen Einzelversuchen genau gleich gehalten werden können. In sämtlichen Versuchen war eine Erhöhung des Grundumsatzes von einer verminderten Phosphorylierung in Zwerchfell und Mitochondrien begleitet.

Zur Lösung der Frage, ob quantitative Zusammenhänge zwischen den beiden Grössen bestehen, wurden sie rechnerisch in Beziehung gesetzt. Geht man von der Annahme aus, dass eine verschlechterte Energiebilanz, wie sie sich in einem verminderten P : O Quotienten ausdrückt, durch eine vermehrte Verbrennung von Nährstoffen kompensiert werden muss, so lässt sich die zu erwartende Erhöhung des Grundumsatzes als Summe einer konvergierenden geometrischen Reihe berechnen. Die prozentuelle Verminderung des P : O Quotienten wird als Anfangsglied dieser Reihe eingesetzt. So errechnet sich z. B. aus einer Erniedrigung des P : O Quotienten von 30 % eine Erhöhung des Grundumsatzes von 42.8 %.

$$\left( 30 \times \frac{1}{1 - \frac{30}{100}} \right)$$

Umgekehrt kann man natürlich auch vom Grundumsatz ausgehend die geforderte Aenderung des P : O Quotienten berechnen. Eine solche Rechnung lässt eine ganze Reihe von Faktoren unberücksichtigt, die Einfluss auf den Grundumsatz haben können. Wie ein Vergleich der experimentell gefundenen mit den aus den P : O Quotienten berechneten Erhöhungen der Grundumsätze zeigt, besteht jedoch tatsächlich in erster Annäherung eine quantitative Uebereinstimmung zwischen den beiden Werten, wenigstens in der Mehrzahl der Versuche. Bei der Beurteilung derselben muss berücksichtigt werden, dass die Fehlerbreite der Bestimmungen, insbesondere des Grundumsatzes, nicht unerheblich ist und sich im ungünstigsten Falle nicht weniger als 4 Fehler addieren können. Die Versuche haben jedoch noch ein anderes Ergebnis gezeigt: die Einwirkung des Thyroxins

TABELLE I

| Versuchstier | Zugeführte Thyroxin (mg./kg.) | Grundumsatz (ml. $O_2$/100 g./Stunde) | | P : O Quotient | | Grundumsatzsteigerung | | |
|---|---|---|---|---|---|---|---|---|
| | | Vorperiode | Nach Zufuhr von Thyroxin | Zwerchfell | Mitochondrien | Gefunden % | Berechnet aus P : O Quotienten (%) Zwerchfell | Berechnet aus P : O Quotienten (%) Mitochondrien |
| Meerschwein | — | 75.4 | 76.6 | 2.76 | 2.62 | | | |
| | 3.6 | 74.2 | 102.0 | 2.00 (—27 %) | 2.0 (—23 %) | 37.5 | 37 | 30 |
| Meerschwein | — | 81.3 | 83.0 | 2.62 | 2.51 | | | |
| | 4.3 | 80.5 | 132.2 | 1.54 (—41 %) | 1.60 (—36 %) | 64 | 70 | 56 |
| Ratte. . . . | — | 103 | 97 | 2.27 | 1.75 | | | |
| | 3.44 | 118 | 202.5 | 1.28 (—44 %) | 1.25 (—29 %) | 72 | 79 | 46 |
| Ratte. . . . | — | 95.7 | 92.5 | 2.24 | 1.97 | | | |
| | 3.34 (*) | 99.5 | 168 | 1.43 (—36 %) | 0.61 (—59 %) | 69 | 56 | 144 |

(*) Trijodthyronin.

auf die oxydative Phosphorylierungsrate verläuft bei Mitochondrien und bei Zwerchfell nicht immer in gleicher Stärke. Besonders in Fällen, bei denen die Abnahme des P : O Quotienten im Zwerchfell geringer war, als sich aus der Erhöhung des Grundumsatzes errechnen liess, zeigten die Lebermitochondrien eine vergleichsweise zu hohe Verminderung der Phosphorylierung. Ein exakter Vergleich zwischen Grundumsatzerhöhung und Verminderung des P : O Quotienten würde demnach eine Bestimmung des Letzteren in sämtlichen Organen eines Tieres voraussetzen und deren Berücksichtigung je nach dem Anteil, den die einzelnen Organe am gesamten Stoffumsatz haben. Die bisherigen Versuche lassen es als wahrscheinlich erscheinen, dass wenn diese methodisch allerdings nur schwierig durchzuführende Voraussetzung erfüllt wird, sich tatsächlich in allen Fällen exakte, quantitative Beziehungen zwischen Grundumsatz und Phosphorylierungsrate ergeben werden.

Eine weitere Versuchsreihe diente zur Klärung der Frage, ob sich entsprechende Zusammenhänge auch bei Ausschaltung der normalen Produktion des Schilddrüsenhormones durch Thyreoidektomie oder Anwendung thyreostatischer Mittel nachweisen lassen. Das ist tatsächlich der Fall, besonders bei Verwendung von Thiouracilen (14). Die auf orale oder parenterale Applikation derselben zu beobachtende Erniedrigung des Grundumsatzes ist in allen Fällen von einer Erhöhung des P : O Quotienten im Zwerchfell begleitet, dessen Ausmass wiederum in einem angenähert quantitativen Verhältnis steht zur Verminderung des Grundumsatzes. In den Lebermitochondrien findet man allerdings Abweichungen, unter Umständen sogar Erniedrigungen des P : O Quotienten. Hierbei spielen ganz offensichtlich in der Literatur mehrfach abweichende Angaben über die Höhe der P : O Quotienten bei Verwendung gleicher Substrate angegeben worden. Es erscheint nicht unwahrscheinlich, dass sich diese Diskrepanzen durch die Verwendung von Versuchstieren verschiedener Entwicklungsstufen erklären lassen. Aus den geschilderten Versuchen lässt sich folgendes Bild der Wirkung des Schilddrüsenhormones ableiten : bei Zufuhr von aussen oder endogen vermehrt durch Ueberfunktion der Thyreoida bewirkt es eine Senkung des P : O Quotienten, der bei sehr starker Dosierung bis auf die Hälfte fallen kann. Auch beim normalen, euthyreotischen Tier führt die ständige Abgabe des Hormones ans Blut in allen Fällen zu einer Senkung des P : O Quotienten unter den theoretisch möglichen Maximalwert; der Quotient jüngerer Tiere ist niedriger als derjeniger von ausgewachsenen, alten Tieren. Eine Annäherung an den theoretisch möglichen Maximalwert erreicht man durch Ausschaltung der Produktion des Schilddrüsenhormones. Diese Angaben basieren auf der Voraussetzung, dass die P : O Quotienten, die man mit isolierten Mitochondrien oder Organteilen *in vitro* misst, ihre Entsprechung in den Verhältnissen *in situ* finden. Diese Annahme wird durch die Tatsache, dass Grundumsatz und P : O Quotient in einem festen gesetzmässigen Verhältnis stehen, gestützt. Auf die Folgerungen, die sich hieraus für eine Erklärung der bei Hyper- oder Hypothyreoidismus beobachteten klinischen Erscheinungen ableiten lassen, soll hier nicht näher eingegangen werden. Dem mit physiologisch-chemischen Zusammenhängen Vertrauten liegen sie auf der Hand, besonders für die Erklärung hyperthyreotischer Zustandsformen. Etwas grössere Schwierigkeiten bieten die Folgeerscheinungen des Thyroxinmangels dem

TABELLE II

| | Grundumsatz (ml. $O_2$/100 g./St.) | P : O Quotient (Zwerchfell) | Grundumsatzsteigerung | |
|---|---|---|---|---|
| | | | gefunden (%) | berechnet (%) |
| Nach 18 × 60 mg. Propylthiouracil, i. p. | 96 | 2.79 | | |
| Kontrolle | 120 | 2.19 (—21.5 %) | 25 | 23 (*) |
| Nach 23 × 60 mg. Propylthiouracil, i. p. | 136 | 2.72 | | |
| Kontrolle | 162 | 2.33 (—14.3 %) | 19.1 | 16.7 (*) |

(*) Diese Werte sind auf das Thiouraciltier als « Normaltier » bezogen.

toxische Wirkungen der Uracile eine Rolle, welche sich besonders in der Leber zu akkumulieren scheinen. Solche toxischen Wirkungen auf Atmung und Phosphorylierung lassen sich auch *in vitro* nachweisen. Die Tabelle II zeigt representative Beispiele solcher Versuche.

Eine weitere Versuchreihe diente der Untersuchung der Beziehung zwischen Grundumsatz und P: O Quotienten bei jungen wachsenden und alten ausgewachsenen Ratten (15). Auch hier wieder ergaben sich die gleichen reziproken Verhältniswerte von Grundumsatz und Phosphorylierung. Junge Tiere mit höherem Grundumsatz zeigen eine niedrigere Phosphorylierungsrate. Es sind Versuch einer kausalen Erklärung. Hierbei wirkt sich der primäre Effekt des Thyroxinmangels offenbar über ein viel komplizierteres System von Folgereaktionen aus.

### *Die Wirkung des Thyroxins* in vitro

Wie wir ebenfalls bereits in unserer ersten Mitteilung zeigen konnten, bewirkt Thyroxin auch *in vitro* eine Herabsetzung des P:O Quotienten in Lebermitochondrien. Das Gleiche wurde auch in Zwerchfellstückchen gefunden (12). Zum Nachweis dieser Wirkung hatte es sich

als notwendig erwiesen, relativ hohe Konzentrationen des Wirkstoffes ($10^{-4}$ — $10^{-5}$ M) anzuwenden und ausserdem die Mitochondrien oder Zwerchfellstücke einige Zeit vor dem Versuch bei 0°C. zu inkubieren. Wie spätere Versuche gezeigt haben (16, und unveröffentlichte Versuche) kommt man jedoch ohne die Präinkubationszeit aus und erzielt auch mit noch geringeren Konzentrationen ($10^{-5}$-$10^{-6}$M, und noch niedriger) nachweisbare Wirkungen auf den P : O Quotienten, die ausserhalb der Fehlerbreite der Versuche liegen. Entscheidend für den Ausfall solcher Versuche ist offenbar die von Tierart zu Tierart und auch von Tier zu Tier verschiedene Empfindlichkeit gegenüber dem Schilddrüsenhormon. Die eingangs geschilderten Versuche über den Einfluss des Schilddrüsenhormones *in vivo* lassen ein solches Verhalten auch erwarten. Wenn bei einem Tier bereits eine gewisse Entkopplung von Atmung und Phosphorylierung durch Beladung der Mitochondrien mit Thyroxin vorliegt, so wird eine noch weitergehende Entkopplung relativ höhere Wirkstoffkonzentration erfordern als bei Tieren, bei denen dieses nicht in dem Masse der Fall ist, da wie schon länger bekannt ist, zwischen verschiedenen Dosen von Thyroxin und der dadurch erzielten Wirkung auf den Grundumsatz nicht arithmetische, sondern logarithmische Beziehungen bestehen (16).

Man darf bei der Beurteilung der *in vitro* Versuche nicht vergessen, dass nach Zufuhr doch schon sehr massiver Dosen Thyroxin *in vivo* die darauf zu beobachtende Erniedrigung des P : O Quotienten meistens unter 50 % liegt. Eine solche Herabsetzung der Phosphorylierungsrate entspricht bereits einer Steigerung des Grundumsatzes um 100 %. Eine 90 % ige Entkopplung, welche man *in vitro* durch entsprechende Konzentrationen von Thyroxin erreichen kann, würde einer Steigerung des Grundumsatzes um 900 % entsprechen. Das sind natürlich völlig unphysiologische Grössenordnungen.

Bei der Interpretation der *in vitro* Versuche ist weiterhin zu berücksichtigen, dass das Hormon in diesen Versuchen innerhalb sehr kurzer Zeit zur Wirkung kommen muss, während *in vivo* die Hormonwirkung erst mit erheblicher Verzögerung eintritt. Neuere Versuche von Logan und Lein (17) haben übrigens gezeigt, dass man mit empfindlicher Technik bereits nach 4 Stunden eine signifikante Erhöhung des Grundumsatzes nachweisen kann. Diese verspätete Reaktion *in vivo* und die relativ hohe wirksame Minimalkonzentration *in vitro* lassen sich mit einer einzigen Voraussetzung erklären, wenn man annimmt, dass das Thyroxin eine gewisse Zeit braucht, um an den Ort seiner Wirkung im Innern der Mitochondrien zu gelangen. Ein solches Verhalten kann von einem Stoff, der drei polare Gruppen enthält und in seinen Löslichkeitseigenschaften eine merkwürdige Mittelstellung zwischen wasserlöslichen und lipoidlöslichen Stoffen einnimmt, ohne weiteres erwartet werden. Es muss indessen zugegeben werden, dass die bisherigen Versuche die Möglichkeit noch nicht völlig ausschliessen, dass das Thyroxin in den Mitochondrien eine Umwandlung in einen noch wirksameren Stoff erst erfährt. Andererseits besteht nach den bisher publizierten Versuchen keineswegs ein zwingender Grund für eine solche Annahme. Bestimmungen der Konzentration von Thyroxin in Mitochondrien nach Einwirkung *in vivo* bzw. *in vitro* kann man nicht ohneweiteres zur Entscheidung dieser Frage heranziehen, da *in vitro* offenbar das Thyroxin zunächst vorwiegend an der Oberfläche adsorbiert wird und wir nicht feststellen können wieviel davon wirklich an den Ort seiner Wirkung gelangt.

Die Wirkung des Thyroxins *in vitro* entspricht weitgehend der des Dinitrophenols. Arbeitet man ohne einen Phosphatakzeptor, so erfolgt eine erhebliche Steigerung des Sauerstoffverbrauches bei gleichzeitig veringertem Einbau von anorganischem Phosphat in ATP. Durch die Lösung der Kopplung von Atmung und Phosphorylierung wird die erstere stimuliert. Bei Gegenwart einer aktiven Hexokinase und von genügend Glukose bleibt die Wirkung auf die Atmung aus, bzw. es kann unter Umständen sogar eine leichte Hemmung des Sauerstoffverbrauches eintreten. Eine solche ist aber nicht obligat und wird als Nebenwirkung bzw. als Zeichen einer Ueberdosierung betrachtet werden können. Der lipoidlösliche Wirkstoff Thyroxin zeigt natürlich andere Relationen von Dosis und Wirkung als das wasserlösliche Dinitrophenol. Der Gesichtspunkt der Verschiedenheit der physikalisch-chemischen Eigenschaften mit allen daraus sich ergebenden Konsequenzen darf überhaupt bei einem Vergleich der pharmakologischen Wirkung beider Stoffe nicht ausser Acht gelassen werden wie es häufig geschieht. Es ist bekannt, dass das Dinitrophenol bei an Myxödem leidenden Patienten zwar den Grundumsatz erhöht, das äussere Erscheinungsbild des Kranken (Hautschwellung usw.) jedoch unbeeinflusst lässt (18). Hieraus auf eine prinzipielle Verschiedenartigkeit des Angriffspunktes von Thyroxin und Dinitrophenol schliessen zu wollen (10), geht jedoch m. E. zu weit. Auch in der Einwirkung auf die Kaulquappenmetamorphose unterscheiden sich beide Stoffe, da nur Thyroxin die Umwandlung der Larven beschleunigt (19). Es konnte jedoch von Méhes und Berde (20) gezeigt werden und eigene Untersuchungen haben es bestätigt, dass Dinitrophenol ebenso wie auch Dikumarol in nicht toxischen Dosen nicht zu einer Erhöhung des Stoffwechsels führt. Letztere muss aber als Veraussetzung für eine Beeinflussung der Metamorphose betrachtet werden, denn durch blosse Erhöhung der Aussentemperatur lässt sich bekanntlich eine beschleunigte Metamorphose bei Axoloteln erzielen bzw. durch eine Herabsetzung dauernd verhindern. Man wird aus den geschilderten Versuchen den naheliegenden Schluss ziehen dürfen, dass zwei Stoffe auf isolierte Enzymsysteme völlig gleichartig wirken und trotzdem im lebenden Organismus sich infolge verschiedener Verteilung abweichend verhalten können. Das Dinitrophenol ebenso wie das Dikumarol kommt offenbar nur in wenigen Organen zur Wirkung, vor allem wohl in der Leber. Dem gegenüber stellt das Thyroxin gewissermassen ein « physiologisches Gift » dar, dessen Verteilung und Wirkungsdauer Gesetzen folgt, die bei normaler Ausschüttung eine harmonische Steuerung der Lebensvorgänge garantieren, während es bei Ueberproduktion aber doch zur Thyreo-« toxikose » kommen kann.

Als Reaktion auf eine Zufuhr von Thyroxin an lebende Versuchstiere ist von vielen Untersuchern eine vermehrte oder verminderte, meist vermehrte, Bildung verschiedener Fermente gefunden worden (eine Literaturzusammenstellung siehe bei 21). Da es sich dabei um solche handelt,

die irgendwie am Stoffwechsel oder der Gewebsatmung beteiligt sind, lag es nahe, in einer Beeinflussung der Synthese solcher Fermente den Angriffspunkt des Schilddrüsenhormones zu sehen. Aber schon die Gleichartigkeit der verschiedenen Befunde kennzeichnet sie insgesamt als sekundäre Folge einer ganz andersartigen Primärreaktion, die nur die Beeinflussung der oxydativen Phosphorylierung sein kann. Wie sollte die Erhöhung der Konzentration irgendeines solchen Enzymes etwa die Erscheinung des Basedow erklären können, wo ja doch das primär Entscheidende nicht die erhöhte Umsatzrate irgendeines Metaboliten sein kann. Angriffspunkt der hormonellen Steuerung kann nur ein Glied der Enzymketten sein, die mit der Produktion verwertbarer Energie betraut sind, das Stoffliche kann in jedem Fall nur folgen. Die einzige Möglichkeit, über die Anderung der Konzentration oder Aktivität eines Enzymes den Energiestoffwechsel steuernd zu beeinflussen, wäre gegeben, wenn es sich um Enzyme handelte, die Verbindungen mit energiereichen Bindungen unter Freisetzung von Wärme hydrolytisch spalten wie z. B. die ATP asen. Die vermehrte Bildung aller anderen Enzyme wird man als adaptive Fermentneubildung auffassen dürfen.

*Die oxydative Phosphorylierung und der Mechanismus der Entkopplung*

Die Frage nach dem Angriffspunkt des Thyroxins führte zwangsläufig zu dem Problem des Mechanismus der Atmungskettenphosphorylierung selbst. Dieses Problem ist noch nicht allzulange in den Gesichtskreis der biochemischen Forschung getreten. Durch die Arbeiten von Engelhardt (22), Kalckar (23), Colowick *et al.* (24) Belitzer und Tsibakova (25), Ochoa (26) und andere, war der Nachweis eines solchen Prozesses erbracht worden und die folgenden Arbeiten verschiedener Laboratorien hatten zur Aufstellung bestimmter Relationen zwischen Sauerstoffverbrauch und Zahl der gebildeten energiereichen Phosphatbindungen geführt (P : O Quotient). Es herrscht heute Uebereinstimmung darüber, dass ein P : O Quotient von maximal 3 erzielt werden kann, wenn man reduzierte Codehydrasen als Wasserstoffdonatoren einsetzt, während man von Bernsteinsäure ausgehend nur einen Quotienten von 2 erhält, von reduziertem Cytochrom *c* schliesslich nur von 1. Die älteren Vorstellungen über die Entstehung energiereicher Phosphorsäurebindungen gingen davon aus, dass eine solche sich vollzieht, wenn die Wasserstoffatome bzw. Elektronen uber die Glieder der bekannten Atmungskette (Codehydrasen, Flavoproteine, Cytochrome) zum Sauerstoff hin wandern. Diese Vorstellung bedarf jedoch eine Ergänzung in dem Sinne, das parallel zu dieser bekannten Enzymkette eine weitere Enzymkette geschaltet ist, bei deren Durchlaufen erst die Phosphorylierungsvorgänge stattfinden. Als Glieder dieser Kette fungieren zwei bekannte Chinon-/Hydrochinonredoxpaare nämlich das Vitamin K und das Vitamin E, beziehungsweise Tokochinon.

Die Beteiligung des Phyllochinons an der Atmungskettenphosphorylierung wurde wahrscheinlich gemacht durch die Beobachtung, dass Stoffe mit Antivitamin K Charakter in gleicher Weise wie Thyroxin und Nitrophenole « entkoppelnd » wirken können (27). Es sind eine grosse Anzahl derartiger Stoffe synthetisiert worden, die sich alle vom 4-Oxycumarin ableiten. Eine Reihe von ihnen sind als Antikoagulantien in klinischem Gebrauch, andere finden als Rodentizide Verwendung in der Schädlingsbekämpfung. Ohne Ausnahme wirken sie auf isolierte Mitochondrien oder Zwerchfellstücke im Sinne einer Entkopplung von Atmung und Phosphorylierung ein. Der wirksame Konzentrationsbereich ist zum Teil recht eng, in höheren Konzentrationen tritt zur Hemmung der Phosphorylierung eine Hemmung der Atmung. Zwischen der Wirkung auf die Phosphorylierung und derjenigen auf die Bildung von Gerinnungsfaktoren (Prothrombin, Faktor VII, usw.), besteht soweit Angaben über Letztere vorliegen annähernd Parallelität. Das führt dazu, die Antikoagulationswirkung dieser Stoffe als sekundäre Folge der primär erfolgenden Wirkung auf die Atmungskettenphosphorylierung zu betrachten und zwar in dem Sinne, dass durch die Hemmung der Energie liefernden Phosphorylierung die Energie verbrauchende Synthese der Eiweisstoffe in diesen Zellen gehemmt wird. Eine ähnliche Hemmung der Bildung gerinnungsfördender Stoffe ist übrigens auch bei Hyperthyreosen beobachtet worden, was sich dem entworfenen Bild des Zustandekommens der Wirkung der Antikoagulantien bestätigend einfügt. Letztere wird offenbar nur in einzelnen Organen bei Anwendung *in vivo* gehemmt (Leber, Knochenmark), da Dikumarol auch in hoher Dosierung am Meerschweinchen kaum eine Wirkung auf den Grundumsatz zeigt trotz stärkster Wirkung auf die Blutgerinnung (13). Dieser Befund steht in Uebereinstimmung mit der Feststellung von Lee und Mitarbeitern (28), dass sich markiertes Dikumarol nur in der Leber für längere Zeit hält, aus dem Blut und übrigen Organen hingegen sehr schnell verschwindet. *In vitro* dagegen wirkt Dikumarol auch auf Zwerchfell « entkoppelnd » (13). Ob bei Vitamin K Mangel die Leber zeitlich ebenfalls besonders früh oder besonders intensiv betroffen wird, ist experimentell schwer zu entscheiden, da die Versuchstiere an dem Prothrombinmangel frühzeitig zu Grunde gehen und Vögel, die ja für diese Versuche fast ausschliesslich in Frage kommen kein Zwerchfell besitzen.

Der direkte Nachweis der Beteiligung des Phyllochinons an der Atmungskettenphosphorylierung war leicht zu erbringen (29, 30). Lebermitochondrien von Vitamin K-frei ernährten Küken zeigen eine deutlich verringerte Phosphorylierungsrate verglichen mit Kontrollen normal ernährter Tiere. Durch Zusatz von Vitamin $K_1$ in geeigneter Konzentration ($10^{-5}$ M) zu einer Mitochondriensuspension aus Lebern von K-freien Tieren lässt sich sogar *in vitro* die Wirkung des Vitamin $K_1$ leicht demonstrieren. In dieser Konzentration steigert Vitamin $K_1$ die Phosphorylierung im Durchschnitt aller unserer Versuche um *ca.* 30 %, in einigen Fällen wurden sogar 50 % erreicht. Das ist genau der Wert, den man erwarten kann, wenn man annimmt, dass das Phyllochinon an einer der drei Phosphorylierungsstufen massgebend beteiligt ist. Dass es sich dabei um die erste, den Codehydrasen am nächsten liegende Phosphorylierungsstufe handeln muss, war nach der Lage des stark negativen Redoxpotentiales des Vitamin $K_1$ ($E'_0 = + 363$mV) zu erwarten. Auch die Tatsache, dass mit Succinat als Wasserstoffdonator eine geringere Differenz der Phosphorylierungs-

raten beim Vergleich des Normaltieres mit dem K-freien Tier beobachtet wird, als mit β-Oxybutyrat (29), spricht für eine solche Einstufung des Phyllochinons. In diesem Falle gelangt der Wasserstoff gleich in die zweite Phosphorylierungsstufe und umgeht die erste.

Eine Beteiligung des Phyllochinons am Wasserstofftransport auf seiner ersten Stufe setzt eine Reaktion zwischen den reduzierten Formen der Codehydrasen (DPNH) und der oxydierten Form(DPN+) des Vitamin $K_1$ voraus. Eine solche wird vermittelt durch eine Phyllochinonreduktase (31), die sich aus Mitochondrien isolieren lässt und welche Wasserstoff von DPNH auf Phyllochinon überträgt. Die Oxydation des gebildeten Phyllohydrochinons erfolgt durch das Cytochrom *b*, wodurch die Stellung des Vitamin K in der Atmungskette eindeutig festgelegt ist. Das lässt sich spektroskopisch leicht nachweisen (32).

Ein durch Cytochrom *c* und Phyllochinonreduktase verstärktes Succinoxydase-Präparat aus Herzmuskel zeigt auf Zusatz von reduzierten DPN momentan die Banden der reduzierten Cytochrome *c* und *a*. Die Bande des reduzierten Cytochrom *b* erscheint nicht. Man hat aus solchen Versuchen bisher den Schluss gezogen, dass das Cytochrom *b* gar nicht auf dem Hauptweg der Atmung läge und nur den von der Bernsteinsäure abgegebenen Wasserstoff übernähme (33, 34). Die Reduktion des Cytochrom *b* tritt aber ein, wenn man dem System eine kleine Menge Phyllochinon zufügt. Damit entfallen alle Einwände, die gegen eine Einschaltung des Cytochrom *b* in die Kette der Atmungsfermente zwischen Codehydrasen und die übrigen Cytochrome bisher zu sprechen schienen (33, 34). Offenbar wird das normalerweise im Gewebe befindliche Phyllochinon bei der Darstellung des Cytochrompräparates heraus gewaschen oder zerstört. Als das wichtigste Ergebnis dieser Beobachtungen ergibt sich die Tatsache, dass es offenbar zwei Alternativwege für den Wasserstoff gibt, die unabhängig voneinander benützt werden können. Nur beim Durchlaufen des einen der beiden wird Energie in Form von energiereichem Phosphat gewonnen. Auf der ersten Stufe der Energiegewinnung ist dies der Weg über das Vitamin K. Ueber den feineren Mechanismus des Zustandekommens dieser energiereichen Bindungen und über die Natur der dabei zweifellos noch mitwirkenden Cofermente oder Wirkgruppen lässt sich zur Zeit noch nichts aussagen.

Die geschilderte Auffassung vom Vorhandensein zweier unabhängiger Wege des Wasserstofftransportes führt zu einer neuartigen Auffassung vom Mechanismus der sogenannten « Entkopplung ». Wenn es durch irgendeinen Mechanismus gelingt, den einen mit einer Phosphorylierungsreaktion gekoppelten Weg für den Wasserstoff zu versperren und ihn so auf den anderen Weg zu zwingen, muss sich als Resultat eine Atmung ohne Phosphorylierung ergeben. Das scheint in der Tat der Fall zu sein. Wie sich durch spektroskopische Verfolgung der Reduktion von Phyllochinon durch DPNH und Phyllochinonreduktase leicht zeigen lässt, lässt sich diese Reaktion durch entkoppelnd wirkende Stoffe wie Thyroxin oder Dikumarol *in vitro* hemmen. Auch die Reduktion des Cytochrom *b* in dem oben beschriebenen Fermentsystem wird durch diese Stoffe verzögert. Es wird z. B. die Reduktion von Phyllochinon ($5 \times 10^{-4}$ M) durch DPNH gehemmt durch Dikumarol : $5 \times 10^{-5}$ M zu 95 %, $2 \times 10^{-5}$ M zu 80 %, $1 \times 10^{-5}$ M zu 70 % ; durch Thyroxin : $5 \times 10^{-4}$ M zu 78 %, $1 \times 10^{-4}$ M zu 65 %, $5 \times 10^{-5}$ M zu 52 %.

Es liegt nahe anzunehmen, dass alle diese « entkoppelnden » Stoffe, die ja chemisch miteinander verwandt sind, da sie alle zur Klasse der Phenole gehören, in gleicher oder wenigstens sehr ähnlicher Weise ihre Hemmwirkung entfalten. Dies könnten sie beispielsweise dadurch, dass sie mit der prostetischen Gruppe eines der an der Atmungskettenphosphorylierung beteiligten Enzyme reagieren. Möglicherweise handelt es sich dabei um die Bindung eines Schwermetallatomes durch Komplexbildung mit den phenolischen OH Gruppen. Dann liesse sich vielleicht auch die Beobachtung von Gemmill (35) einordnen, dass sich das Thyroxin durch eine hohe Affinität zu Kupferionen auszeichnet. Wie insbesondere die Versuche mit verschiedenen substituierten Dikumarolen zeigen, müssen aber auch noch andersartige Faktoren, die in der ganzen Struktur des Moleküls zu suchen sind, am Zustandekommen eines hochwirksamen, « entkoppelnd » wirkenden Stoffes beteiligt sein.

Unerklärt bleibt zunächst noch die Tatsache, dass in der normalen unvergifteten Zelle von den beiden möglichen Wegen vom Wasserstoff nur der eine und zwar der mit Energiegewinnung verbundene und damit gewissermassen mühsamerer Weg beschritten wird. Das Wesen der « Entkopplung », das nach der geschilderten Auffassung in einer « Umschaltung » von dem einen auf den anderen Weg besteht, scheint verständlich zu werden, dasjenige der « Kopplung » bleibt noch aufzuklären.

Im Zusammenhang mit dem Problem der Beteiligung des Vitamin K an der Atmung und Atmungskettenphosphorylierung blieb noch eine Frage zu klären. Bekanntlich erweist sich im Tierversuch ausser Vitamin $K_1$ auch das Vitamin $K_2$ und vor allem auch das einfache 2-Methyl-1,4-naphtochinon (Menadion) als gerinnungsfördernd wirksam. Die früher mehrfach vertretene Auffassung wonach das Letztere die eigentlich wirksame Form des K-Vitamins darstellen oder gar die, dass das Menadion Bestandteil des Prothrombins sein solle (vgl. 36), hat in neuerer Zeit weniger Zustimmung gefunden. Die Auffindung der Rolle des K-Vitamins bei der Atmungskettenphosphorylierung und die Zurückführung der antithrombotischen Wirkung auf diese primäre Funktion bot die Möglichkeit, die Frage nach der Natur der wirksamen Form der K-Vitamine experimentell auf neuen Wegen zu überprüfen. Es wurden hierzu im *in vitro*. Test mittelst Lebermitochondrien Vitamin K-frei ernährter Küken verglichen : Vitamin $K_1$ und zwei 2-Methylnaphtochinone, von denen das eine in der 3-Stellung die Gruppe : $-CH_2-CH=C=(CH_3)_2$, das andere die Gruppe : $-CH_2-CH=C(CH_3)-(CH_2-CH_2-CH=C(CH_3))_2-CH_3$ trug (37).

In gleicher molar-Konzentration angewandt ($10^{-5}$ M) bewirkte Phyllochinon die bekannte Erhöhung, die Verbindung mit der Butylen-Seitenkette dagegen eine ausgeprochene Hemmung der Phosphorylierung, wie sie auch bei Zusatz von 2-Methyl-1,4-naphtochinon oder Phtiocol beobachtet wird. Die Verbindung mit der dreifach ungesättigten Seitenkette mit 15 C-Atomen

nahm eine Mittelstellung ein : Sie bewirkte eine deutliche, jedoch geringere Erhöhung der Phosphorylierungsrate wie Vitamin $K_1$.

Diese Versuche stimmen in den Ergebnissen vollkommen überein mit einer Untersuchung von Isler und Mitarbeitern (38), die zeigen konnten, dass sich die experimentelle Dikumarolvergiftung bei Kaninchen und Ratten nur durch substituierte Methylnaphtochinone aufheben lässt, deren Seitenkette eine Länge aufweist, die der der natürlich vorkommenden K-Vitamine einigermassen nahe kommt. Man wird hieraus den Schluss ableiten dürfen, dass die Letzteren die wirksame Form des K-Vitamins darstellen und dass das einfache Methylnaphtochinon im Körper durch Einführung des Phytolrestes in diese wirksame Form übergeführt wird. Es wäre damit als Provitamin-K zu bezeichnen.

Ueber die Bedeutung der Seitenkette wird man sich folgende Vorstellung bilden dürfen : Phyllochinon kommt im Tier in den Mitochondrien, in der Pflanze in den Chloroplasten vor. Beide sind durch ihren hohen Gehalt an Lipoiden ausgezeichnet. Nichts liegt näher als anzunehmen, dass der Phytolrest oder die entsprechende Gruppe des Vitamin-$K_2$ den Zweck hat, die eigentliche Wirkgruppe, das Redoxsystem des Naphtochinons, in einer Struktur-bildenden Grundsubstanz von Lipoidcharakter in geeigneter Position zu verankern. Zu einer solchen Vorstellung passen Beobachtungen, wonach es gelingt, durch geeignete Behandlung von Leberhomogenaten mit Phosphatid spaltenden Lecithinasen das System der Atmungskettenphosphorylierung zu schädigen ohne die Gewebsatmung selbst zu beeinflussen (39).

Das geschilderte neue, in Einzelheiten noch ergänzungs- und verbesserungsbedürftige Bild vom Aufbau des System der Atmungskettenphosphorylierung auf der ersten der insgesamt drei Stufen, musste die Frage nahe legen, ob auch bei den folgenden zwei Stufen nicht mit dem Vorliegen ähnlicher Verhältnisse zu rechnen sei. Insbesondere erschien es wahrscheinlich anzunehmen, dass auch bei diesen Chinon-/Hydrochinonsysteme eine Rolle spielen könnten. Der Verdacht musste sich hierbei vor allen Dingen auf das zweite fettlösliche Vitamin richten, das wie das Vitamin K, ein mit einer Phytolgruppe substituiertes Hydrochinon darstellt, das Vitamin E. Eine Beteiligung des Vitamin E am Phosphatstoffwechsel und an der Gewebsatmung ist schon wiederholt diskutiert worden, und es liegen eine Reihe von Beobachtungen vor, die auf einen Zusammenhang zwischen der Vitamin-E Wirkung und dem System der Adenosinphosphorsäure hinweisen (z. B. 40). Beobachtungen über die Beteiligung des Vitamin-E am Elektronentransport sind kürzlich von Nason (41) publiziert worden. Dass das Vitamin E auch hier eine ganz entsprechende Rolle spielt wie das Vitamin-K, d. h. an einem mit Atmungskettenphosphorylierung verbundenen der bekannten Atmungskette parallel geschalteten Transportweg beteiligt ist, lässt sich leicht nachweisen (42). Misst man die Atmungskettenphosphorylierung in Zwerchfellstückchen normaler und E-frei ernährter Kaninchen, so zeigen die letzteren eine bedeutend niedrigere Phosphorylierungsrate als die ersteren, während die Atmung in beiden annähernd gleich gefunden wird. Verwendet man Succinat als Wasserstoffdonator und hat man bei den Versuchstieren einen hohen Grad von Vitamin E-Freiheit erreicht, kann man bei den Kontrollen eine nahezu doppelt so hohe Phosphorylierung messen wie bei den E-Mangeltieren. Mit β-Oxybuttersäure als Wasserstoffdonator liegen die Werte näher aneinander. Es liegen also gerade die umgekehrten Verhältnisse vor wie bei K-Mangeltieren. Das ist auch zu erwarten wenn man davon ausgeht, dass das Vitamin-E an der zweiten (oder der letzten) Phosphorylierungsstufe mitwirkt.

TABELLE III

*P : O Quotienten (representative Versuche)*

| | Zwerchfell | | Leber-mito-chondrien |
|---|---|---|---|
| | Succinat | β-Oxybutyrat | Succinat |
| Kontrolle . . . . | 1.2 | — | — |
| E-Mangeltier . . | 0.42 (—65 %) | — | — |
| Kontrolle . . . . | 1.3 | 2.5 | — |
| E-Mangeltier . . | 0.95 (—27 %) | 2.3 (—8 %) | — |
| Kontrolle . . . . | 1.25 | 1.61 | 1.33 |
| E-Mangeltier . . | 0.84 (—33 %) | 1.18 (—27 %) | 1.15 (—13 %) |

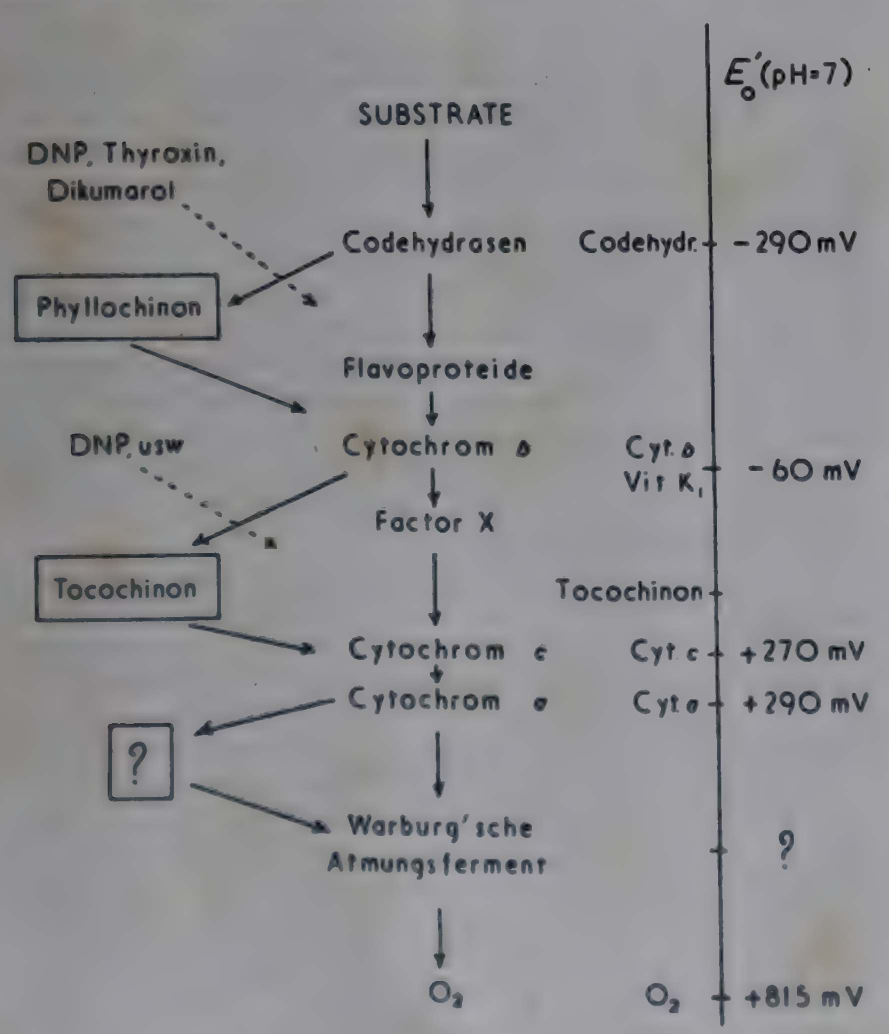
Weiterhin ist für die Verhältnisse bei E-Mangel charakteristisch, dass man in Lebermitochondrien geringere oder gar keine Unterschiede zwischen normal und E-frei ernährten Tieren findet. Entsprechendes haben schon Rabinovitz und Boyer (43) bei Herzhomogenaten festgestellt. Man wird aus diesen Beobachtungen die Vorstellung ableiten dürfen, dass nicht alle Organe in gleicher Weise durch den Vitaminmangel betroffen werden. Das bekanntlich chemisch sehr stabile Vitamin scheint sich lange im Körper halten zu können und wird bis zuletzt mit besonderer Zähigkeit von den lebenswichtigeren Organen festgehalten, während die weniger lebenswichtigen den Vitaminmangel früher zu spüren bekommen. Dazu gehören die Skelettmuskulatur und die Fortpflanzungsorgane.

*In vitro* lässt sich die Wirkung des Vitamin E schwerer nachweisen. In schon seit längerer Zeit durchgeführten Versuchen gelang es durch Zuzatz von α-Tocopherol, α-Tocopheroxyd, α-Tocochinon und α-Tocohydrochinon zu Zwerchfellstückchen E -frei ernährter Tiere eine Erhöhung der Phosphorylierungsrate zu bewirken. Diese Erhöhung betrug jedoch meistens nicht mehr als 10-20 % und liess sich auch nicht mit Sicherheit stets reproduzieren. Es hat den Anschein, als ob der wirksame Konzentrationsbereich besonders eng wäre und dass sich *in vitro* eine Wirkung nur in einem bestimmten Stadium des Vitaminmangels demonstrieren lässt.

Bezüglich der Einordnung des Vitamin-E in ein modifiziertes Atmungskettenschema ist man daher mehr auf Vermutungen angewiesen als beim Vitamin-K. Manches spricht dafür, dass nicht das Vitamin-E als

solches sondern das Tocochinon die wirksame Form darstellt. Dass dieses biologisch ebenso wirksam ist wie das Tocopherol, ist in letzter Zeit mehrfach nachgewiesen worden (44). Allerdings erstreckt sich die biologische Wirksamkeit nur auf die Heilung der Muskeldystrophie bei Kaninchen, nicht dagegen auf die Verhinderung der Resorptionssterilität bei Ratten (45). Das muss aber nicht notwendig einen Einwand gegen die oben geschilderte Auffassung von der biologischen Rolle des Tocochinons darstellen. Da Tocopherol und Tocochinon verschiedene chemische und physikalische Eigenschaften besitzen, so dass z. B. nur das erstere im Organismus gespeichert werden kann, ist eine verschiedenartige biologische Wirkung ohne weiteres verständlich. Die verschiedene Durchlässigkeit der Placenta z. B. könnte eine Erklärung für die unterschiedliche Wirksamkeit der beiden Stoffe im Fertilitätstest abgeben.

Durch Aufnahme von Wasserstoff würde das Tocochinon zu Tocohydrochinon reduziert werden, welches wiederum ganz glatt das Cytochrom *c* reduziert. Man bedient sich ja seit langem des Hydrochinons oder des sich ähnlich verhaltenden Phenylendiamins zum enzymatischen Nachweis des Systems Cytochrom *c*/Cytochromoxydase. Das natürliche Substrat dieses Enzymsystemes dürfte das Tocohydrochinon sein. Der Lage seines Redoxpotentiales nach liegt es zwischen den Cytochromen *b* und *c* und ist um etwa 230 mV positiver als das Phyllochinon. Das wäre aber gerade die Potentialdifferenz, die der Bildung einer energiereichen Phosphatbildung entsprechen würde. Da auch auf der zweiten Stufe der Atmungskettenphosphorylierung die gleichen




Hypothetisches Schema der Atmungs- und Phosphorylierungskette.

Stoffe entkoppeln wie auf der ersten Stufe, was zum Teil in direkten Versuchen gezeigt worden ist, aber auch aus der Tatsache geschlossen werden kann, dass durch alle Entkopplungsgifte die Phosphorylierung fast zu 100 % gehemmt werden kann, wird man in der Annahme eines entsprechenden Umschalt-Mechanismus wie beim Phyllochinon-system zum mindesten eine naheliegende Hypothese erblicken dürfen (vgl. auch 46).

Damit ist man freilich schon in das Gebiet der Spekulation gelangt, in das man sich ganz und gar begeben muss, wenn man versucht, die dritte dem Sauerstoff am nächsten gelegene Stufe der Atmungskettenphosphorylierung zu betreten. Man könnte hier daran denken, dass vielleicht das System Tocopherol/Tocopheroxyd mit seinem positiveren Redoxpotential eingeschaltet ist oder aber vielleicht sogar das Thyroxin, das durch die Substitution mit 2 Jodatomen ein ganz besonders positives Potential besitzen muss. Das würde diesem Wirkstoff noch eine ganz andere Funktion zuweisen als die zu Beginn geschilderte regulierende. Da das Thyroxin in seinem Vorkommen aber auf den Kreis der höheren Tiere beschränkt ist, erscheint eine solche Annahme sehr unwahrscheinlich und würde dazu nötigen, in Pflanzen, Mikroorganismen und niederen Tieren einen anderen Wirkstoff an dieser Stelle zu fordern. Ueber den Wirkungsgrad der Atmungskettenphosphorylierung bei allen diesen Organismen sind wir allerdings bisher ja überhaupt noch völlig im Unklaren und wir dürfen sicherlich noch manche Ueberraschung bei der weiteren experimentellen Durchforschung dieses weiten Gebietes erwarten.

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# Purification and structure of β-corticotropin and its active degradation products (*)

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(*Received* 3 *July* 1955)

The physiological importance of the hormones of the anterior pituitary gland has been the subject of many papers in the last two decades. It was recognized that certain of these materials were involved in the formation of steroids by the adrenal gland. However, it took the announcement that ACTH was effective in rheumatoid arthritis (1) to stimulate research workers to the efforts which have resulted in the appearance of several hundred reports in the literature of the last six to eight years. Based on our current knowledge, the great bulk of this work was carried out using very impure material. For this reason, we do not wish to discuss the physiological functions of corticotropin (ACTH), except as they relate to the chemistry of this hormone.

### *Purification of corticotropin*

Early work by Li *et al.* (2, 3) and Sayers *et al.* (4) led to the conclusion that ACTH was a pure protein with a molecular weight of about 20 000. The peptide character of the active principle was demonstrated by its inactivation by proteolytic enzymes such as trypsin (3, 5) chymotrypsin (5), arginase, tyrosinase and extensive pepsin treatment (3). The active material was insoluble in 5 % trichloroacetic acid (TCA) and in the pH range of 5.5 to 9.0.

During the course of these studies, a satisfactory method of assay was devised by Sayers and his group (6). The method was based on the observation that it was possible to relate the ascorbic acid depletion of the adrenal glands of hypophysectomized rats to the dosage of ACTH administered. This assay, although only semi-quantitative at best, made it possible to follow the course of chemical purification without great difficulty.

Further attempts at purification of the molecular weight 20 000 protein, in our laboratories and others, suggested that a smaller component associated with this protein was responsible for the activity. The reality of this concept was very dramatically demonstrated by the work of Astwood *et al.* (7), who effected a 50-fold purification by adsorbing the activity of either crude preparations or the 20 000 molecular weight protein on oxidized cellulose from 0.1 N acetic acid solutions. Subsequently the activity was eluted from the oxycellulose with 0.1 N hydrochloric acid. This highly active preparation (OC-ACTH) served as starting material for the fractionation, degradation and proof of structure which we are reporting here.

In the early stages of our studies a great many methods of peptide and protein purification were investigated : pH and sodium chloride precipitation ; the formation of insoluble salts ; dialysis ; ultracentrifugation ; electrophoresis ; paper strip chromatography ; partition, adsorption and ion exchange chromatography ; and countercurrent distribution methods (8). Of these methods, countercurrent distribution proved to be the only one which resolved the OC-ACTH mixture into seven corticotropin components and $\delta_1$-intermedin.

In order to use our 200-tube, automatically-driven, all-glass machines most effectively, we investigated some 250 two-phase solvent systems for their ability to distribute the ACTH activity. It was concluded from these studies that the following types of systems could be used for this work : (*a*) phenols *vs.* water (phenols or cresols), (*b*) basic solvents *vs.* water (lutidines), (*c*) butanols *vs.* aqueous acid (TCA, trifluoroacetic, picric, toluenesulfonic, dichloroacetic, monochloroacetic, hydrochloric or acetic acids, in decreasing order of effectiveness), and (*d*) butanols *vs.* aqueous salt solutions. The first two types led to extensive emulsification, which made them impractical for use in the automatic distribution apparatus.

The separation of OC-ACTH which is provided by the *n*-butanol *vs.* 0.5 % TCA system is shown in figure 1. This separation was so clean that it was necessary to carry the distribution only ten transfers to effect complete separation. For recovery, hydrochloric acid and benzene were added to the pooled distribution samples and, after 3-fold extraction, the products were converted to their acetate salts by the use of Amberlite

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(*) This work was done in collaboration with the following colleagues : (Stamford Laboratories) K. S. Howard, A. R. Cacciola, S. B. Davis, D. S. Davies, E. A. Eigner, J. P. English, B. M. Finn, J. H. Meisenhelder, N. E. Shakespeare, S. D. Willson ; (Lederle Laboratories) A. W. Moyer, R. A. Brown, R. G. Child, M. C. Davies, C. C. Scrobola, J. van der Scheer.

The lecture was given on Tuesday 2 August 1955 at a joint session of section 2 (chemistry and physical chemistry of proteins and polypeptides) and section 7 (biochemical regulations), the first part (preparation of β-corticotropin and active degradation products) by P. H. Bell, the second part (determination of structure of β-corticotropin) by R. G. Shepherd.

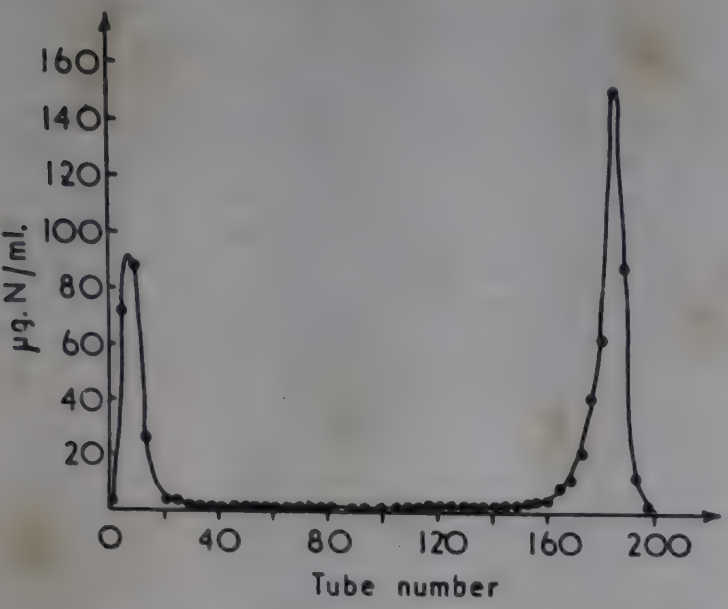


FIG. 1. — Distribution of OC-ACTH; 0.5 % trichloracetic acid *vs.* *n*-butanol; 413 mg. in two tubes; n = 199.

IRA-400 ion exchange resin. The fast component T(6-10) contained 80 % of the weight and 90 % of the corticotropin activity of the starting OC-ACTH. The slow fraction B(1-5) accounted for 20 % of the weight and 10 % of the activity of the charge. Increasing the concentration of TCA in the system led to further fractionation of B(1-5), as shown in figure 2. In this

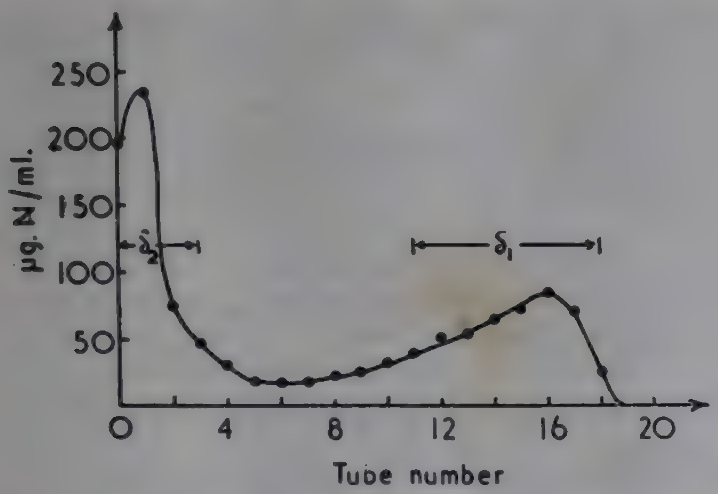


FIG. 2. — Distribution of B(1-5); 2.0 % trichloracetic acid *vs.* *n*-butanol; 504 mg. in one tube; n = 19.

case the $\delta_1$ fraction accounted for approximately one-half of the weight and essentially all of the corticotropin activity of B(1-5). This $\delta_1$ material contains about 90 % of the original intermedin activity. The remaining 10 %, which was another type of intermedin (*vide infra*), was present in T(6-10) and persisted in all samples having corticotropin activity.

Attempts to fractionate T(6-10) using type (*c*) solvent systems were unsuccessful. However, using a type (*d*) solvent system, its components were resolved as shown in figure 3. This experiment suggested that at least six components were present in the T(6-10) fraction. The reality of this separation could not be established, however, until the fractions had been recovered and redistributed.

The recovery of these proteins from distributions containing salts was possible due to the fact that the addition of TCA increased the distribution coefficient of ACTH far more than that of the sodium ion. A four-stage countercurrent extraction completely separated the sodium chloride from the peptide material, which was then recovered in the manner described for the TCA systems.

The components shown in figure 3 were isolated as $\alpha$, $\beta$, $\gamma_1$ and $\gamma_2$ pools as shown, using the technique outlined above. These recovered fractions were then redistri-

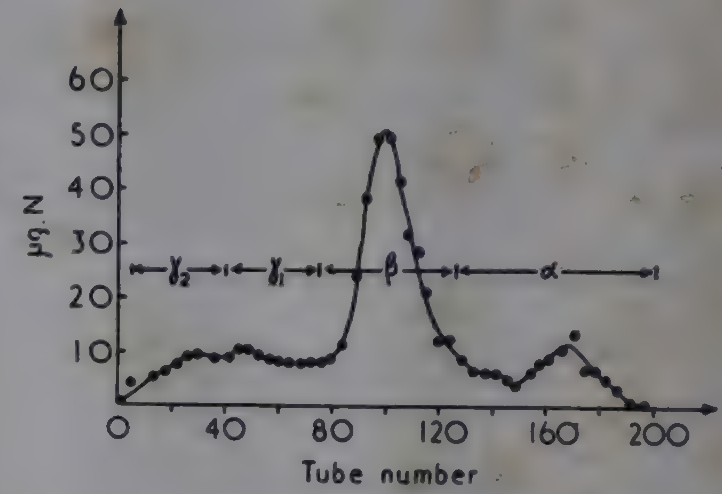


FIG. 3. — Distribution of T(6-10); 3.5 % NaCl + 6.0 % HOAc *vs.* *n*-butanol; n = 207.

buted in the same solvent system, with the results as shown in figure 4. These redistributions were carried out for the number of transfers necessary to obtain adequate resolution. Therefore, to illustrate the agrement with the initial distribution results of figure 3, the concentration has been plotted *versus* the calculated distribution coefficients $K$ (9).

The reality of the initial fractionation was confirmed by these experiments. It can also be seen that the $\alpha$ pool resolved into the three components $\alpha_1$, $\alpha_2$ and $\alpha_3$. The $\beta$ fraction was essentially free of other materials and gave a distribution curve which is nearly theore-

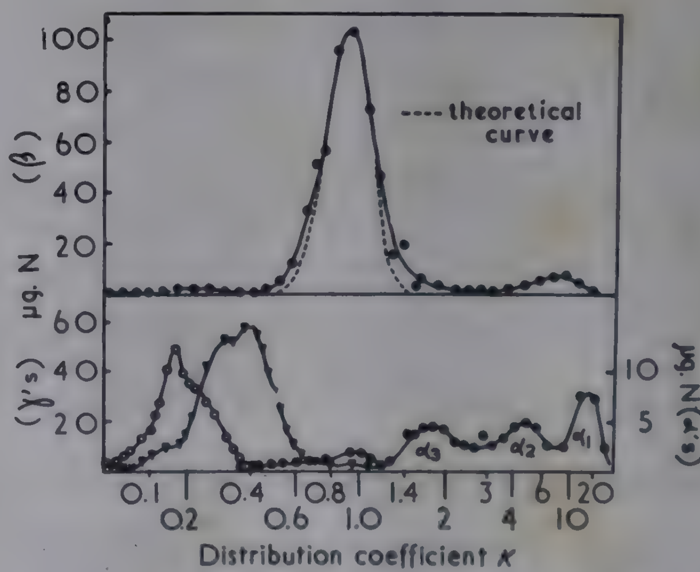


FIG. 4. — Redistribution experiments; 3.5 % NaCl + 6.0 % HOAc *vs.* *n*-butanol.

Upper curve : $\beta$; n = 138.

Lower curve : 0 = $\gamma_1$-pool, n = 411,

▽ = $\gamma_2$-pool, n = 393,

● = $\alpha$-pool, n = 200.

tical (9). On the other hand, the $\gamma_1$ and $\gamma_2$ fractions were both contaminated with a third component which was given the designation of $\gamma_3$. All of these fractions showed nearly equal biological activities by the Sayers' ascorbic acid depletion assay.

At this point it became necessary to select one of the fractions for studies designed to prepare the smallest possible active molecule. The β fraction was selected for the following reasons : (*a*) it represented the largest single active component of the clinical grade ACTH (ca. 35 % of the activity), and (*b*) it digested with pepsin to three easily-separated active products rather than the six to eight products from the other fractions.

Larger quantities of the β-corticotropin for this work were prepared from OC-ACTH using the countercurrent method illustrated in figure 3. After 287 transfers the distribution was analyzed, giving a curve similar to that expected from the earlier studies. At this point the α and γ components were removed from the machine and the tubes from which these fractions were withdrawn were washed and refilled with the solvent system. The machine was set so as to recycle the organic phase for 200 additional transfers, at which point the remaining α and γ material had separated and was removed. The distribution of the β peak was continued until a total of 920 transfers had been applied. The distribution curve of the β peak was then essentially theoretical in shape and distribution coefficients determined by nitrogen analysis were constant for selected tubes across the peak. This β-corticotropin was considered to be homogeneous as far as this method was concerned and was withdrawn, recovered and studied for homogeneity by other methods.

In contrast to the T(6-10) material, β-corticotropin appeared to be homogeneous in the ultracentrifuge. Treatment of the ultracentrifuge data by the method of Archibald (10) gave a value of 4500 for the molecular weight of the free base form of the hormone. Amino acid analysis by the Dowex 50 method of Moore and Stein (11) gave the following minimum amino acid empirical formula : $Ala_3Arg_3Asp_2Gly_3Glu_5His_1Leu_3Lys_4Met_1Phe_3Pro_4Ser_3Try_1Tyr_2Val_3(NH_3)_1$. The minimum formula weight of 4546 derived from this analysis is in excellent agreement with the molecular weight obtained from the ultra-centrifuge studies.

End group studies by the DNP method of Sanger (12) revealed only one N-terminal amino acid (serine) and C-terminal studies using carboxypeptidase suggested a single sequence of-Leu-Glu-Phe·OH.

All these data seemed sufficient to justify the conclusion that the β-corticotropin was essentially homogeneous, and a suitable material for degradation studies.

### *Degradation of β-corticotropin to active products*

Li and his colleagues (3) had reported that crude corticotropin could be degraded by mild pepsin or acid treatment without loss of biological activity. We selected pepsin as an approach to smaller active molecules since preliminary results showed that the resultant mixture was much less complex than that obtained from the less specific acid treatment. Digestion conditions were selected such that the original TCA precipitability had disappeared, indicating that the parent β had all been degraded. Treatment at an enzyme : substrate ratio of 1 : 360 for one hour at 37° C. in 0.01 N hydrochloric acid was sufficient. The pepsin was inactivated by boiling and removed by TCA treatment. The TCA-soluble products were converted into their acetate salts with ion exchange resin and recovered by lyophilization to yield 85 % of the weight of the starting material.

A typical countercurrent separation of these products is shown in figure 5. The slowest major peak was

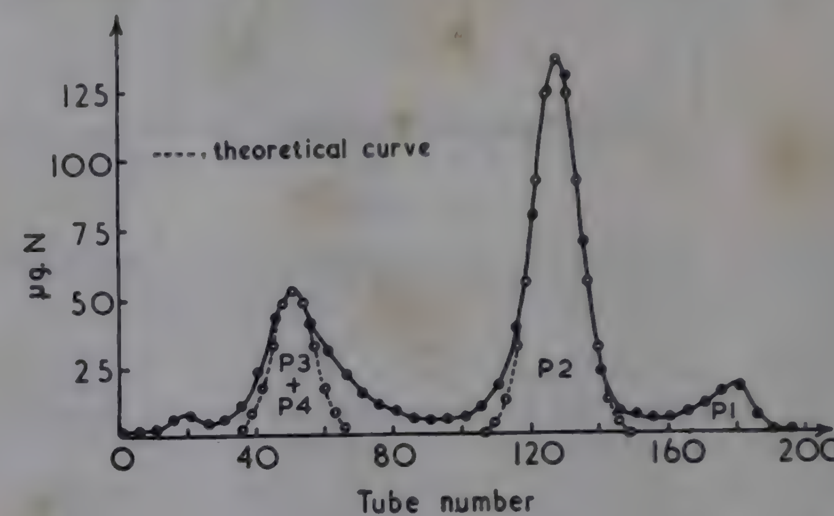


FIG. 5. — Distribution of pepsin-digested β-corticotropin ; 0.5 % trichloracetic acid *vs.* *n*-butanol.

heterogeneous as indicated by its deviation from the (dotted) theoretical curve. This mixture was resolved by clearing the material from tubes 80-200 and carrying out an extended distribution in which the organic phase was recycled. In figure 6 the analysis of the material

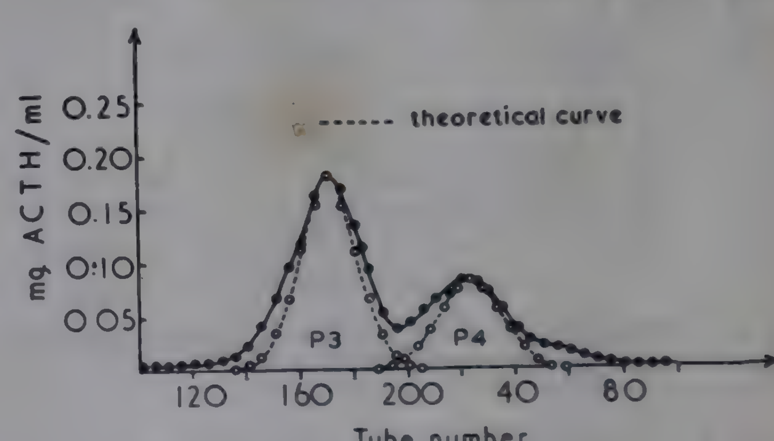


FIG. 6. — Continued distribution of P3 + P4 ; 0.5 % trichloracetic acid *vs.* *n*-butanol ; n = 921.

after 900 transfers shows two components to be present in this peak. The proportions of these products were varied by changing the enzyme to substrate ratio, as shown in table I. The amounts are expressed in % of the total material charged in the distribution.

TABLE I

| Enzyme : substrate | P1 | P2 | P3 + P4 |
|---|---|---|---|
| 1 : 40 | 14 % | 13 % | 73 % |
| 1 : 120 | 15 % | 33 % | 52 % |
| 1 : 360 | 11 % | 51 % | 38 % |

P2 could be an intermediate form between either P3 or P4, or both. This point was settled by a distribution of redigested P2, which degraded to P4 only, as shown in figure 7. These data suggest that the digestion proceeds in the following manner :

$$\beta \xrightarrow{\textit{pepsin}} \text{P2 or P3} + \text{small peptides}$$
$$\downarrow \textit{pepsin}$$
$$\text{P4} + \text{small peptides}$$

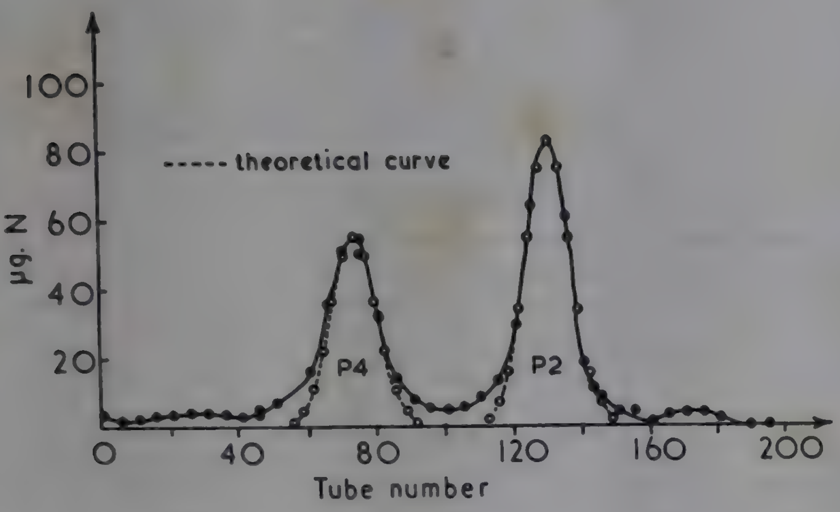


Fig. 7. — Distribution of pepsin-digested P2; 0.5 % trichloracetic acid *vs.* *n*-butanol, n = 199.

The relation of the minor product P1 to this mechanism has not been determined, but P1 seems clearly not involved in the predominant process shown.

P2, P3 and P4 all had the same qualitative and quantitative activity as β, as measured by the Sayers' ascorbic acid depletion assay. These products and P1 account for approximately 85 % of the weight of the starting β-corticotropin. The remaining 15 % was small acidic peptides which had been cleaved off by pepsin. These peptides had been removed by the ion-exchange resin in the pepsin digestion recovery process.

Comparison of the composition of P2, P3 and P4 with the parent β peptide revealed the nature and number of amino acids which were lost during the pepsin digestion as shown in table II.

Table II

| Moles of amino acid lost | In conversion of β to | | |
|---|---|---|---|
| | P2 | P3 | P4 |
| Leucine | 1 | 2 | 2 |
| Phenylalanine | 2 | 2 | 2 |
| Proline | 1 | 1 | 1 |
| Alanine | 2 | 2 | 2 |
| Glutamic Acid | 2 | 2 | 3 |
| Aspartic Acid | | | 1 |

The structures of these small acidic peptides, given in table III, were determined by methods which will be described later. These data established the C terminal sequence of β-corticotropin up to the P4 breakpoint, as illustrated below :

Table III

| Peptide | Composition |
|---|---|
| PA2 | H·Pro-Leu-Glu-Phe·OH |
| PA1 | H·Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe·OH |
| PA3 | H·(Ala,Glu,Ala,Phe,Pro,Leu,Glu)OH |
| PA4 | H·Leu(Ala,Glu,Ala,Phe)·OH |
| (1) | H·Glu-Leu·OH (Glu —$NH_2$) |
| (2) | H(Asp)Glu-Leu·OH (Glu —$NH_2$) |

-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe·OH

↑ P4 ↑ P3 ↑ P2

C-terminal sequence of β-corticotropin

P4 is the smallest active molecule which has been isolated from peptic digests. More extensive pepsin treatment (13) results in the cleavage of a pentapeptide from the N-terminal chain under conditions causing inactivation. Further work was directed toward structure elucidation in order to investigate the feasibility of synthesis and the relation of activity to other portions of the peptide chain.

### *Degradation to inactive products*

β-corticotropin was selected for structural studies since preparation of the smaller P4 involved considerable time and low yields. In addition, the known 11 amino acid C-terminal end was not expected to complicate this work.

Partial acid hydrolysis as an approach to the structure did not appear promising for two reasons. The very large number of products would present an extremely complex fractionation problem. In addition, the limitations imposed by the quantities of material available made imperative the choice of a method which was more selective than random hydrolysis.

The presence of seven basic and six aromatic amino acid residues in β-corticotropin suggested the use of trypsin and chymotrypsin. These enzymes would be expected to cleave the peptide chain at different points and to preserve special structural features which would be destroyed by partial acid hydrolysis. A small sample was likely to be sufficient, in view of the anticipated specific degradation to only a small number of products. The structure of the parent molecule would readily follow from the overlapping at the different cleavage points.

Possible disadvantages of the enzymatic method are the greater amount of sequence determination on the products, as compared to partial acid hydrolysis and the chance of rearrangements catalyzed by the enzymes. The sequence work was feasible with the newer methods. In order to rule out transpeptidation such as reported by Waley and his associates (14, 15), minimal exposure to the enzymes was used and a quantitative separation of the products of the enzymatic degradation from the two different enzymes was carried out. Furthermore, the two sets of major products from both enzymes each accounted for all the amino acids in β and each led to the same consistent structure for β.

*Trypsin digestion.* — Digestion with this enzyme rapidly led to inactivation of β-corticotropin. Preliminary studies showed that a minimal degree of digestion of six to eight peptides resulted from a 4-hour digestion at pH 7.7 and 25° C., using an enzyme : substrate ratio of 1 : 100. These conditions gave a mixture of peptides which could be separated by extended countercurrent distribution and paper chromatography in *n*-butanol *vs.* 20 % acetic acid. This solvent system, although it was not ideal for the separation of the basic peptides in the mixture, had the advantages of volatility and absence of salt, and thus involved no possible recovery losses. In figure 8 is shown the distribution of the trypsin digest

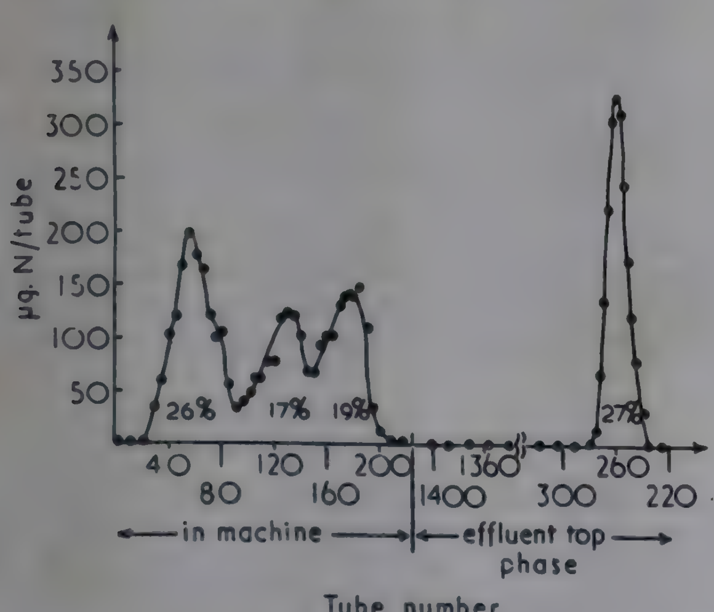


FIG. 8. — Distribution of trypsin-digested β-corticotropin; *n*-butanol *vs.* 20 % acetic acid; n = 1400.

after 1400 transfers. This distribution was continued to a total of 8200 transfers in order to resolve the slow-moving basic components. Complete separation of these peptides was finally achieved by paper chromatography. The weight yields, as well as the distribution coefficients, are shown in table IV. Total weight recovery was 93 %

*Chymotrypsin digestion.* — Chymotrypsin digestion also led to rapid inactivation of β-corticotropin. With this enzyme a 24-hour digestion at pH 7.7 and 25° C., and a 1 : 100 enzyme : substrate ratio, was used. This digest mixture was also separated by distribution in *n*-butanol *vs.* 20 % acetic acid. In this case 9500 transfers were applied and final separation by paper chromatography

TABLE IV

*Peptides from trypsin and chymotrypsin digestions of β-corticotropin*

| Digestion with | Peptide | Calc. K | Weight recovered | % of theory |
|---|---|---|---|---|
| Trypsin (200 mg.) | T1 | 6.0 | 74 mg. | 96 |
| | T10 | 0.14 | 38 | 97 |
| | T14 | 0.10 | 34 | 96 |
| | T17 | 0.04 | 30 | >50 |
| | T15, T16, T18, T19 | 0.05 | 10 | minor products |
| | Total . . . . . | | 186 mg. | 93 |
| Chymotrypsin (179 mg.) | C2 | 0.9 | 11 | 100 |
| | C4 | 0.4 | 10 | 100 |
| | C5 | 0.3 | 11 | >85 |
| | C7 | 0.2 | 24 | 96 |
| | C10 | 0.06 | 59 | *ca.* 50 |
| | C16 | 0.01 | 16 | 86 |
| | Total . . . . . | | 131 mg. | 73 |

was necessary to complete the purification of the most basic components. The peptides found, along with their weight yields and distribution coefficients, are also shown in table IV. In this case the overall weight recovery was only 73 % since several minor peptides from the experiment were not examined for composition or recovered in a weighable form. Subsequent studies on the peptides shown in table IV revealed their structures and made it possible to express the recovered weights in terms of the theoretical yields shown.

*Structural studies of degradation products of β-corticotropin*

The trypsin and chymotrypsin degradation products listed in table IV were considered suitable for structural study only when they had satisfied the following criteria : (1) homogeneity by countercurrent distribution and paper chromatography, (2) single C- and N-terminal groups, and (3) amino acid analyses giving integral mole ratios. Amino acid analyses of the acid hydrolysates of these peptides were carried out using one- or two-dimensional paper chromatography with solvents suited to the composition of each peptide. The resolved amino acids were quantitated by elution of their ninhydrin color from the paper. This eluted color was compared with individual standard curves for each amino acid since their color responses vary widely. The good operating range of this procedure was 0.5-2.0 × $10^{-7}$ moles of hydrolyzed peptide.

Most of the peptide sequence methods were studied and their limitations determined. These studies led us to select the Edman method (16) for N-terminal sequence and carboxypeptidase for C-terminal sequence work. These were adapted to use on a milligram scale and to quantitative paper chromatography.

The thiohydantoins obtained from the Edman stepwise degradation were removed by ethyl acetate extraction and regenerated to the amino acids by barium hydroxide hydrolysis for identification and quantitative estimation. The yields were generally low (20-40 %) due to the occurrence of a side-reaction during the hydrochloric acid-acetic acid cyclization. Frequently more than one amino acid spot was found in the Edman end group hydrolysate but possible confusion was avoided by quantitative comparison of the spots. The end group was then easily recognized by being present in 5- to 10-fold the amount of the other spots. Serine, arginine and tryptophan fail to appear as an end group by this Edman procedure and their locations are based on these 'blanks' and data from other methods.

The C-terminal sequence was derived from quantitative paper chromatography of aliquots after appropriate time intervals of exposure of the peptide to carboxypeptidase. Since this analysis required the absence of salt, a volatile combination of ammonium bicarbonate and ammonium acetate was used for buffering and for increasing the enzyme solubility. The relation of the amounts of the amino acids, and their approach to molar proportions, permitted the assignment of a definite sequence.

Wherever possible, the Edman and carboxypeptidase methods were applied to each peptide until the derived sequences overlapped. The results of these structural studies are summarized in table V. The correct amino acid sequence for each peptide is indicated by the Brand system of abbreviation (17). The portion of the structure of certain peptides in parentheses was not determined directly from a study of the peptide in question.

The overlapping of the trypsin, chymotrypsin and pepsin degradation products makes it possible to fit together an amino acid sequence of β-corticotropin (18). The group of products from trypsin and from chymotrypsin each accounts for all the amino acid content of the parent molecule and they lead to sequences which are consistent. The sequence of the (Asp,Gly,Ala, Glu) portion at positions 25-28 was not derived from the sequence work on the trypsin product T1, due to instability to the Edman acid cyclization conditions in stages 4, 5 and 6 of the degradation. Carboxypeptidase failed to digest this same portion at the end of P4.

Obviously the simplest fragment containing this » unknown » portion would be the heptapeptide carboxyl end of P4 which would be split off by trypsin. From a trypsin digest of P4, this P4T1 degradation product ($K = 0.39$) was separated by a 500-transfer countercurrent distribution in *n*-butanol *vs.* 20 % acetic acid. Determination of its sequence by the Edman method failed in the same manner as before and carboxypeptidase again failed to digest the carboxyl end. The acid lability mentioned made partial acid hydrolysis especially promising because of the unimolar ratio of amino acids. The parent peptide was completely destroyed by two hours' treatment at 105° C. with 0.1 N hydrochloric acid and a maximum amount of intermediate peptides were formed with a minimum of amino acid formation. Paper chromatography in *n*-butanol : water : acetic acid (5 : 4 : 1) was used to isolate peptides I, II, IV and VI of table V. The determination of the amino acid sequence of these peptides permitted assigning a sequence to P4T1 and this completed the sequence of β-corticotropin (19) as shown in figure 9.

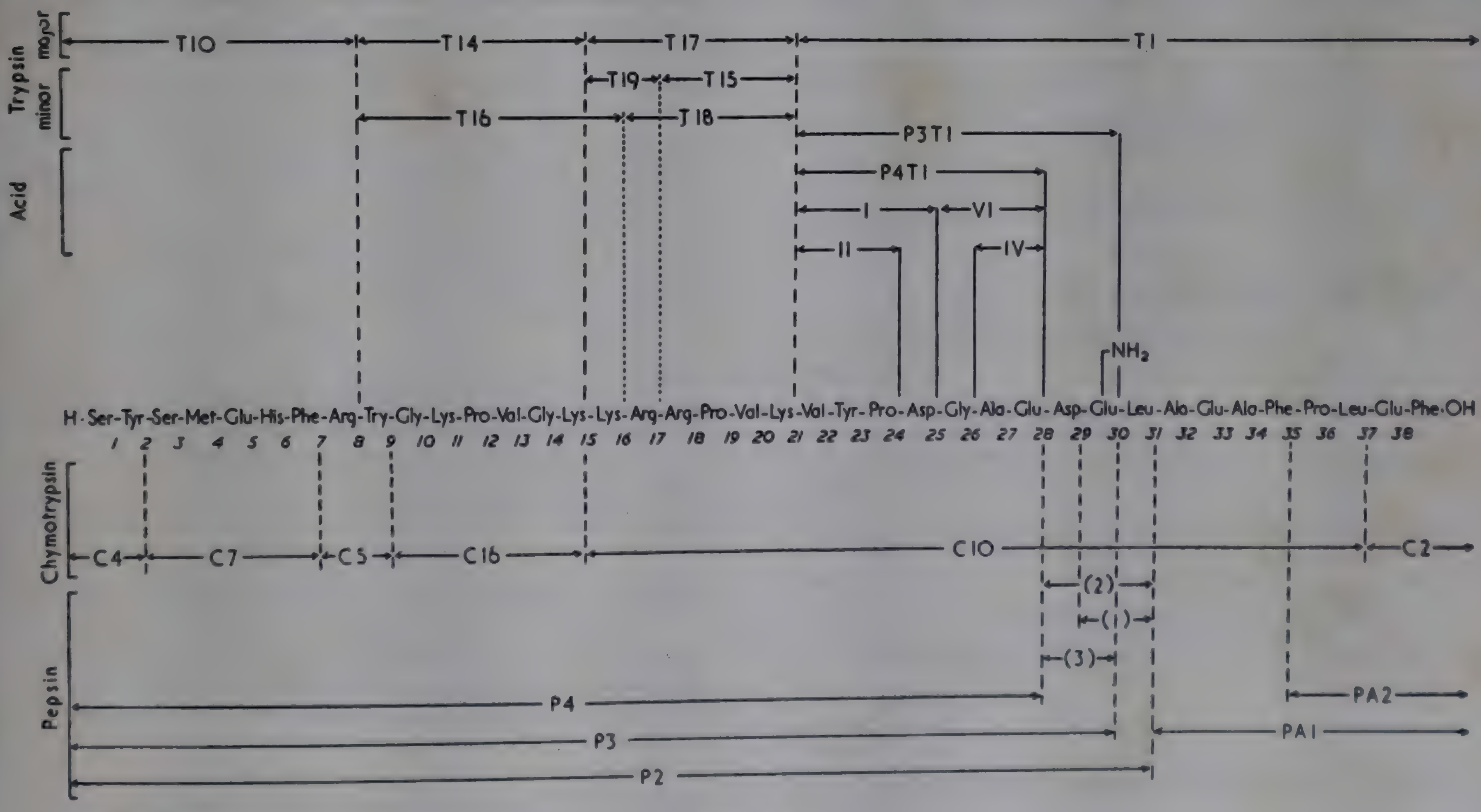


FIG. 9.

TABLE V

*Degradation products of β-corticotropin*

| Peptide | Structure |
|---|---|
| | TRYPSIN AND CHYMOTRYPSIN |
| C4 | H·Ser-Tyr·OH |
| C7 | H·Ser-Met-Glu-His-Phe·OH |
| T10 | H·Ser-Tyr-Ser(Met,Glu,His)Phe-Arg·OH |
| C5 | H·Arg-Try·OH |
| T14 | H·Try-Gly-Lys-Pro-Val-Gly-Lys·OH |
| T16 | H·(Try,Gly,Lys,Pro,Val)Gly-Lys-Lys·OH |
| C16 | H·(Gly,Lys,Pro,Val,Gly,Lys)·OH |
| T19 | H·(Lys,Arg)·OH |
| T18 | H·(Arg,Arg,Pro)Val-Lys·OH |
| T15 | H·(Arg,Pro)Val-Lys·OH |
| T17 | H·Lys-Arg-Arg-Pro-Val-Lys·OH |
| | —$NH_2$ |
| C10 | H·Lys-Arg-Arg-Pro-Val(Lys,Val,Tyr,Pro,Asp,Gly,Ala,Glu,Asp,Glu,Leu,Ala,Glu,Ala,Phe,Pro)Leu·OH |
| | —$NH_2$ |
| T1 | H·Val-Tyr-Pro(Asp,Gly,Ala,Glu,Asp,Glu,Leu,Ala,Glu,Ala,Phe,Pro)Leu-Glu-Phe·OH |
| C2 | H·(Glu,Phe)·OH |
| | PEPSIN |
| | —$NH_2$ |
| (3) | H·(Asp,Glu)·OH |
| | —$NH_2$ |
| (1) | H·Glu-Leu·OH |
| | —$NH_2$ |
| (2) | H·(Asp,Glu,Leu)·OH |
| PA4 | H·Leu(Ala,Glu,Ala,Phe)·OH |
| PA2 | H·Pro-Leu-Glu-Phe·OH |
| PA1 | H·Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe·OH |
| | PEPSIN, TRYPSIN AND ACID |
| P4T1 | H·Val-Tyr-Pro(Asp,Gly,Ala,Glu)·OH |
| II | H·Val-Tyr-Pro·OH |
| I | H·Val-Tyr-Pro-Asp·OH |
| VI | H·Gly-Ala-Glu·OH |
| IV | H·Ala-Glu·OH |
| | —$NH_2$ |
| P3T1 | H·(Val,Tyr,Pro,Asp,Gly,Ala,Glu,Asp)Glu·OH |

### *Structure of β-corticotropin*

The question of alternate linkage or of branching has been considered in the case of each polyfunctional amino acid. Branching at the lysine ε-amino groups is ruled out by the DNP reaction of β and by the Edman degradation of the lysine peptides. The possibility of alternate linkages for aspartic and glutamic acids must be considered in all peptide degradations. The Edman degradation ruled out the possibility of non-α peptide linkages for all such bonds except 25, 28 and 29. Application of the Dakin (20, 21) reaction to peptide P4T1 indicated that bond 25 was formed by the α-carboxyl group of the aspartic acid. The Dakin reaction gave equivocal results with P3T1 and leaves bonds 28 and 29 uncertain in this respect. The cleavage of bond 28 by pepsin suggests an α-linkage.

To complete the detailed structure of the hormone the optical configuration of each amino acid was investigated. Microbiological analyses (*) of each resolved amino acid from an acid hydrolysate of corticotropin agreed very closely with the chemical analyses. This established the L configuration for all of the amino acids except tryptophan (destroyed by acid) and alanine and aspartic acids (test not specific for L alanine and L aspartic acid).

### *Relation of structure to biological activities*

*Corticotropin and adipokinin.* — The corticotropin and adipokinin (22) assay results (23) for peptides P2, P3 and P4 were as high as for β-corticotropin. From this fact it can be concluded that the 11 amino acids from bonds 28-38 are not necessary for these activities. Acid digestion of β using conditions more drastic than those required to split completely bond 24 in peptide P4T1 did not destroy corticotropin activity. This observation suggests that amino acids 1-24 comprise the minimum peptide structure necessary for corticotropin activity. Lack of activity in peptides T10, T14, T17 and C10 suggests that a major portion, if not all, of the 1-24 amino acid sequence is needed for corticotropin action. In addition, alkali inactivation of β splits the first, second and third bonds and conditions causing pepsin inactivation split the fifth (13). Thus, the amino end cannot be altered without inactivation, while the carboxyl end can be changed very drastically.

The effect of mild alkali treatment is the removal of the amide from the glutamine at position 30 and we believe that the corticotropin-A of White *et al.* (13) is the deamidated form of the natural hormone.

*Intermedin (Melanophore expansion activity).* — Two types of intermedin activities were found in corticotropin preparations (18) and readily separated :

(*a*) High activity which is not increased by alkali treatment ($\delta_1$ fraction is of this type).

(*b*) Low activity which is potentiated by alkali treatment. All corticotropin fractions (except $\delta_1$) and the active degradations products P2, P3 and P4 fall in this class.

The constancy of the type (*b*) intermedin to corticotropin activity ratio for preparations with such widely different histories strongly suggests that the minimum structural requirements for the type (*b*) intermedin activity are to be found within the 1-24 amino acid sequence of the β-corticotropin structure. Intermedin activity is rapidly destroyed by trypsin and chymotrypsin and no such activity was present in any of the fractions of these enzymatic digests.

Hot dilute alkali rapidly destroys the corticotropin and adipokinin activities with simultaneous 30- to 100-fold increase in the intermedin activity of type (*b*) material. 90 % of the intermedin activity of alkali-treated material was different by countercurrent distribution from $\delta_1$ although the remaining 10 % had a distribution coefficient like that of $\delta_1$. However, the chemical relation of the two types of intermedin has not been further studied. The presence of the original $\delta_1$-intermedin could not be due to alkaline degradation during isolation since only acidic media were used.

(*) We are grateful to Mr. A. C. Dornbush for the microbiological analyses.

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# Biochemical properties of plastides (*)

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(*Received* 1 *August* 1955)

Certain important principles have been established in the study of the biochemical properties of protoplasm structures carried out in the past ten years. It has been found that many enzymatic reactions in the living cell are associated with structural components of the cell. The most studied constituents of living cells are the chloroplasts by the properties of which is determined the specific nature of the plant.

According to the concept prevalent in plant physiology and biochemistry, approximately ten to fifteen years ago, the role of chloroplasts in plant cells was limited to their photosynthetic activity. Only after the method of differential centrifugation had been widely adapted for experimental purposes it has been possible to study various biochemical properties of plastides.

A thorough investigation of the biochemical properties of plastides has shown that many enzymatic systems of the cell are associated with them.

I should like to report on the most important results of the investigations on the biochemical properties of plastides in our laboratory, carried out in collaboration with A. M. Kobyakova, I. I. Filippovich, M. S. Odintsova, B. P. Smirnov and I. M. Mosolova. Our investigations have shown that the enzymatic action of plastides depends both on the nature and physiological condition of the plant organism and on the mode of attachment of the enzymes with the protein complexes of the plastides.

### *Dependence of enzymatic activity of plastides on the specific nature of an organism*

Our investigations have shown that the action of enzymes in plastides depends on the resistance to storage of the plant. It appears that the action of the lipoxidase in the chromoplasts of two different varieties of carrots differs in the same storage conditions (table I).

No traces of lipoxidase activity have been found in chromoplasts isolated from carrots resistent to storage (OA 515) from the beginning of storage, down to the end of February. The action of enzymes becomes evident in April with a sharp intensification in May. In varieties which are not resistent to storage (Nantes) the activity of lipoxidase in chromoplasts is evident right at the storage beginning; gradually weaking, it drops to zero values at the end of the storage period (February).

TABLE I

*Lipoxidase activity in chromoplasts of carrots (in µl. $O_2$/g. dry weight/15 min.)*

| Date of experiment | Variety | |
|---|---|---|
| | Nantes | Moskovskaya zimnaya OA 515 |
| November 15 . . . . . . | 276 | 0 |
| December 7 . . . . . . | 227 | 0 |
| February 12 . . . . . . . | 0 | 0 |
| April 5 . . . . . . . . . | — | 182 |
| May 10 . . . . . . . . . | — | 435 |

It should be pointed out that in identical experimental conditions, high lipoxidase activity is observed in the chromoplasts of the resistent variety of carrots after the autolysis, which is apparently due to changes in the linkage of the enzyme with the protein complex of the chromoplasts.

### *Nature of linkage between enzymes and protein complex of plastides*

Subsequent investigations revealed that most enzymes in various types of plastides are bound to the structural components of the plastides; moreover, the mode of the bond changes in the ontogenetic development and under the action of various agents. Disturbance in the resting state and etiolation of plants weakens the bond between the enzyme and protein complex of plastides.

As the result of etiolation and even of the keeping of green plants for several days in darkness one can

(*) Lecture given on Tuesday 2 August 1955 at a joint session of section 5 (intermediary metabolism) and section 11 (plant biochemistry and biochemistry of the soil). The text of this lecture has already been published, in Russian and in French by the U. S. S. R. Academy of Sciences, Moscow 1955.

observe a considerable increase in the free and loosely bound enzymes. Photosynthesis, on the contrary, tends to stabilize this bond.

The bond between the enzymes and the protein complex of plastides may be destroyed altogether by creating a high osmotic concentration, by means of a lengthy autolysis, dehydration, freezing, by the action of various solvents and so on.

As established by our investigations, labilization of the bond of enzymes with structural components of plastides during storage is observed also in the depository organs of plants.

Table II shows the change in the bonds of the invertase in sugar beet leucoplasts after four months storage, as compared with leucoplasts isolated at the beginning of the storage time.

TABLE II.

*Changes in the invertase bond with the protein complex of the plastides*

| Mode of the bond | Invertase activity | |
|---|---|---|
| | September | December |
| Free | 14.28 | 33.58 |
| Loosely bound | 32.25 | 43.54 |
| Firmly bound | 53.35 | 32.88 |

Results are expressed in % of total invertase activity.

This table indicates that after four months storage a substantial change takes place in the properties of the invertase, the quantity of free and loosely bound enzymes is increased, and that of the firmly bound enzymes declined.

Later investigations have shown that the capacity of proteins to form the complexes with lipoids, nucleic acids and with the protein complex of plastides is determined in many respects by the same factors which cause changes in the bond of various other enzymes with the same complex. Thus, the ageing leads to changes in the amounts of bound lipoids, in the quantity and quality of ribonucleic acid, in the protein compounds of plastides, and simultaneously in the stability of the bond of various enzymes with the protein complex of plastides. It is, therefore, natural to assume that reasons for the changes in the stability of enzymes bond with the plastide should be sought in the alterations of the general complex-forming capacity of plastide proteins.

The possibility that the bonds of enzymes with the structural components of plastides tend to weaken under the action of various factors should be taken into account in evaluation of the enzymatic action of cell structures. It is therefore, hardly possibly to agree with the opinion of those investigators who draw their conclusions from the enzymatic properties of one or another structural component of the protoplasm, the chloroplasts for example, on the results of experiments carried out without account of this fact. It should also be taken into account that the labilization of enzyme bond with the cell structure may cause not only the transition of enzymes into solution, but also their interchange between cell structural components.

The existence of many enzymatic systems in plastides, the comparatively high content of protein and nucleic acids indicates that these cell structures play an exceptionally important part in various biochemical functions of cells, in synthetic processes as well.

### *Cyclophorase system of chloroplasts*

Of great interest is the investigation of the cyclophorase enzymes in plants. There are references about the great lability of the cyclophorase system in living cells. Perhaps many efforts to detect this system in plant tissues have failed, owing to its lability in plants as well, although some relations of this catalytic system to the tricarboxylic cycle have already been established in various plants.

Our investigations of the cyclophorase system in isolated chloroplasts have encountered fairly great difficulties and many of these obstacles are still existing.

Nevertheless, we have succeeded in showing that in definite experimental conditions chloroplasts isolated from the autumn leaves of sugar beet and from bean leaves oxidize some acids of the Krebs cycle. The results of an experiment on chloroplasts of beans (figure 1) show that fumaric, citric and succinic acids

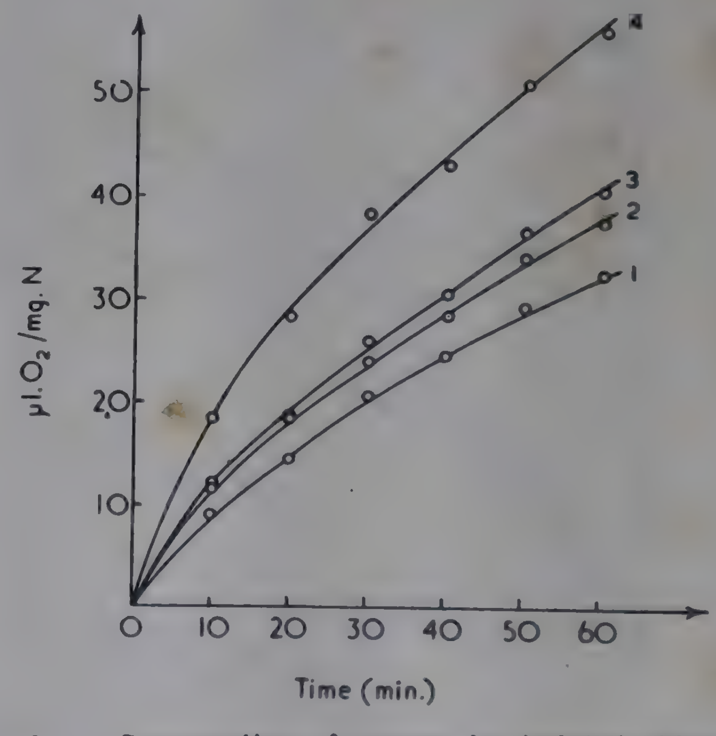


FIG. 1. — Consumption of oxygen by isolated chloroplasts of beams : (1) chloroplasts, (2) chloroplasts + succinate, (3) chloroplasts + citrate, (4) chloroplasts + fumarate.

are oxidized by isolated chloroplasts. These and the earlier data as yet are inadequate for a conclusion about the extent of the cyclophorase system occurrence in plants, about its localization and role in some structural constituents of the plant cell.

Investigations on the role of chloroplasts in the synthetic functions of the plant cell have been carried out in our laboratory along the following three lines :

(*a*) study of the synthesis of fatty acids and incorporation of radio-phosphate in chloroplast phospholipids ;

(*b*) determination of the connection between the amount of ribonucleic acid and one of the proteins in chloroplasts ;

(*c*) study of the peptide bond synthesis in isolated chloroplasts.

### *Synthesis and oxidation of fatty acids ; ³²P incorporation in chloroplast phospholipides*

The chemistry of the synthesis and transformation of fatty acids, which has been thoroughly investigated in the animal organism, is practically unexplored in plant tissues. Adequate informations on the role of plant cell structural constituents in the transformation of fatty acids is still lacking.

Experiments carried out in our laboratory have shown that higher fatty acids constitute about 55-60 % dry weight of the lipoid fraction of isolated chloroplasts. It appears that chloroplasts possess a very pronounced capacity for oxidizing both unsaturated and saturated fatty acids, such as linoleic, linolenic, oleic and palmitic acids.

Figure 2 shows the results of experiments on oxydation of fatty acids by chloroplasts of bean leaves which have revealed that chloroplasts possess not only lipoxidase action connected with the oxidation of linoleic and linolenic acids but they contain also an enzymatic system which cause a deeper oxidation of such fatty

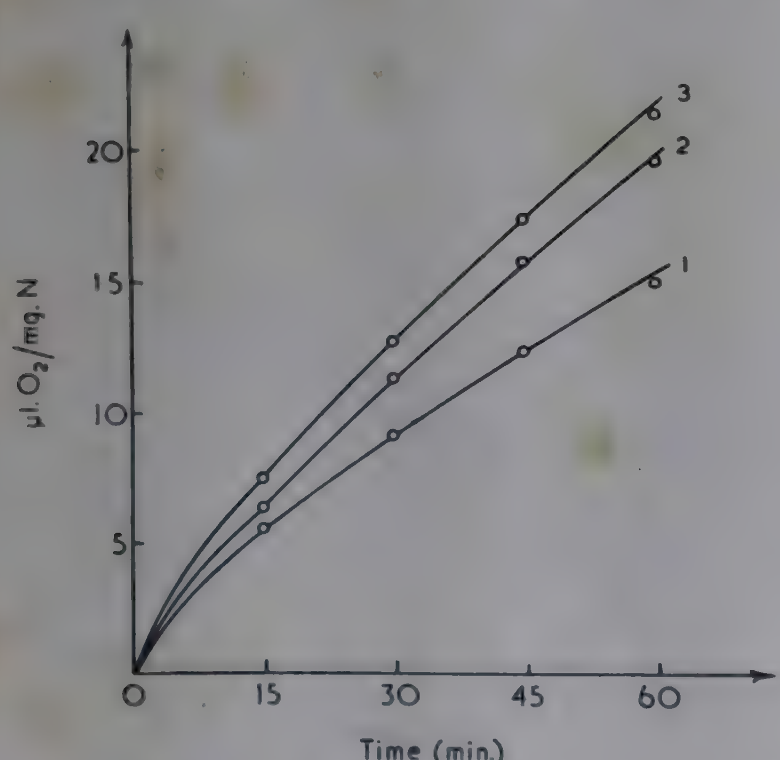


Fig. 2. — Oxidation of fatty acids by isolated chloroplasts : (1) palmitic acid, (2) oleic acid, (3) linoleic + linolenic acid.

acids as the oleic and palmitic acids. The chemical mechanism is still unknown. Our efforts to stimulate these reactions by adding diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) were not successful, although it is known that identical experiments with plant mitochondria have shown the importance of these compounds for oxidation processes.

Later investigations have shown that the incubation of chloroplasts isolated from the sunflower shoots with radioactive acetate leads to the incorporation of labelled acetate in the higher fatty acids of the lipoid fraction (table III).

Table III.

*Incorporation of radioactive acetate into higher fatty acids of chloroplasts*

| Expt. nr. | c.p.m. | μg. acetate incorporated |
|---|---|---|
| 1 | 1075 | 26.2 |
| 2 | 1340 | 21.4 |

Results are expressed per 100 mg. of fatty acids.

The addition of ATP does not affect the acetate incorporation. It should be pointed out that chloroplasts isolated from bean leaves have not been found to incorporate acetate into their fatty acids. The observed differences could not be explained on the basis of the experiments. The chloroplasts isolated from the bean leaves may have perhaps a weaker system for catalysing the acetate incorporation into the fatty acids, than the corresponding system of the chloroplasts isolated from the sunflower seed lobe ; but the possibility is not excluded that this difference is determined by the specific nature of metabolism in these plants.

It has also been established that phosphorus is incorporated into the phospholipids during the incubation of chloroplasts with radioactive phosphorus (table IV).

Table IV.

*Incorporation of ³²P into phospholipids of bean chloroplasts*

| Expt. nr. | c.p.m. | μg. ³²P incorporated |
|---|---|---|
| 1 | 5590 | 4.28 |
| 2 | 7910 | 6.08 |

Results are expressed per 100 mg. of phospholipides.

Table IV gives the results of experiments on the incorporation of ³²P into the phospholipids of chloroplasts isolated from ten days old bean shoots. This process takes place only in the presence of adenylic acid ; it suggests the possibility that phosphorus may be incorporated into phospholipids *via* ATP.

### *Ribonucleic acid content and its relationship with the quantity of proteins in chloroplasts*

Our investigations have established that plastides isolated from the leaves of sugar beet, spinach, sunflower and tobacco, as well as from the roots of sugar beet and carrots contain, ribonucleic acid in varying amounts from 0.5 to 3.5 % dry weight, depending on the type and age of the plant. It has also been established that during the development of the plant, the amount of ribonucleic acid (RNA) in chloroplasts, just as in other biological objects is subjected to regular changes. Chloroplasts of young leaves contain two or three times

as much RNA as chloroplasts isolated from the old leaves. And it is characteristic that a qualitative change in RNA composition occurs simultaneously (table V).

TABLE V.

*Purine and pyrimidine bases content of the RNA of the chloroplasts of tobacco leaves during budding (arbitrary units)*

| | Guanine | Adenine | Purine : pyrimidine |
|---|---|---|---|
| Upper tier. . . . . | 1.08 | 1 | 1.64 |
| Middle tier . . . . | 1.22 | 1 | 1.30 |
| Lower tier. . . . . | 1.31 | 1 | 1.27 |

It is seen from table V, that as the leaves grow older the guanine : adenine ratio increases and the purine : pyrimidine ratio becomes smaller in RNA extracted from tobacco chloroplasts, *i. e.* the pyrimidine content of RNA seems to increase. The increase of pyrimidine bases in RNA was established also in ageing sugar beet chloroplasts. It should be pointed out also that the relation between nitrogen bases of the chloroplast RNA is not equimolecular. That is apparently due to the fact that the RNA of chloroplasts does not possess a tetranucleotide structure.

Of great interest are the parallel changes in the RNA and protein content in chloroplasts observed during the process of plant development. As it is seen in figure 3, the decrease in the amount of RNA as the plant ages is accompanied by a reduction of protein content in the chloroplasts of sugar beet leaves.

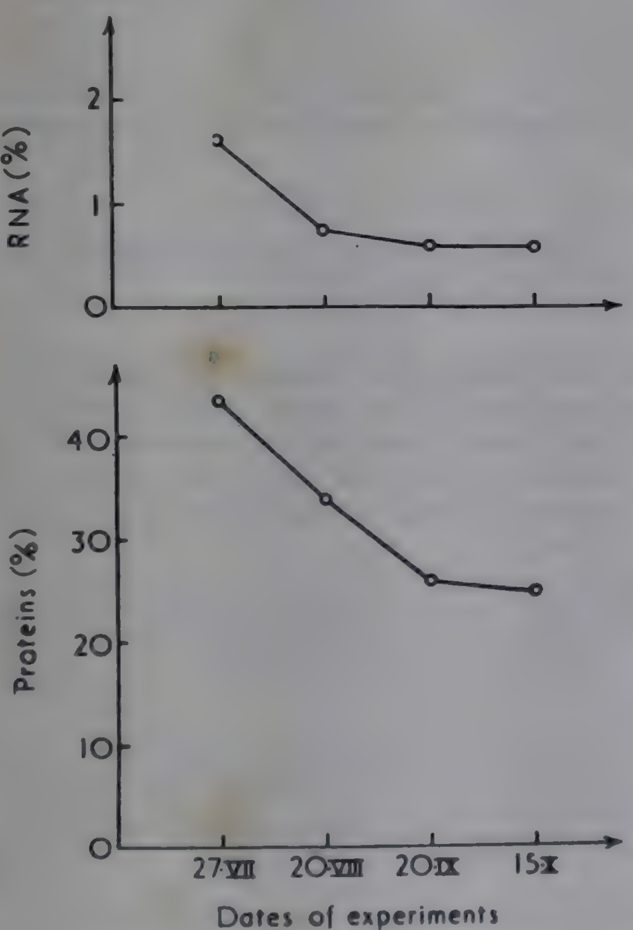


FIG. 3. — Ribonucleic acid (RNA) and proteins in chloroplasts during the development of plants.

It will also be of interest to note that the decrease in the amount of RNA is accompanied by an increase in the activity of the chloroplasts' ribonuclease. Thus, for example, if the content of RNA in the chloroplasts of sugar beet leaves equals 0.8 % in the fourth rosette, the ribonuclease activity is 5.91 (mg. of P/100 mg. N.). An identical experiment carried out with chloroplasts of the twelfth rosette leaves revealed that when the content of RNA is raised to 2.1 % while the ribonuclease activity drops to 2.14 (mg. of P/100 mg. N.).

The facts observed, as well as the widespread opinion that nucleic acids take part in the synthesis of proteins, lead us to the assumption that a synthesis of peptide bonds takes place in the chloroplasts.

### *Synthesis of proteins in chloroplasts*

The hypothesis that chloroplasts are involved in the synthesis of the peptide bonds was confirmed by a series of experiments carried out in our laboratory. It has been proved that the incubation of chloroplasts of clover with a mixture of amino-acids containing glycocolle, threonine, serine, tyrosine, valine, alanine, arginine, lysine, aspartic and glutamic acids in proportions corresponding to those in the proteins of plastides does not lead to any noticeable change in the amount of protein nitrogen. But without added aminoacids the amount of protein nitrogen increases at the expense of non-protein nitrogen ; moreover, no decrease has been revealed in the amount of free aminoacids determined by the ninhydrin method. It suggests that the observed increase in protein nitrogen is due perhaps to the action of free peptides.

Later experiments have shown that in fact the amount of protein nitrogen in chloroplasts increases in the presence of added di- and tripeptides. It is evident from figure 4, that the incubation of sugar beet chloroplasts with glycyl-glycine in the presence of ATP, succinate

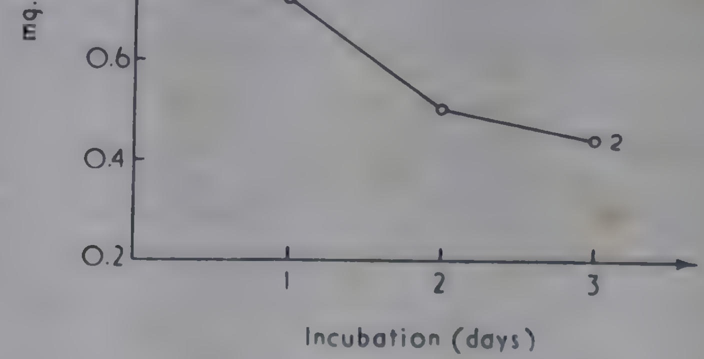


FIG. 4. — Changes in contents of protein and non-protein nitrogen during the incubation of chloroplasts with dipeptides and ATP : (1) protein nitrogen, (2) non-protein nitrogen.

and fumarate leads to an increase in protein nitrogen and to a reduction of non-protein nitrogen. The increase of protein nitrogen represents approximately 12 % of the total amount of nitrogen in the chloroplasts. Such changes in the 'protein N : non-protein N' ratio may be observed only in the presence of ATP.

The same phenomenon was observed but to a smaller degree during the incubation of chloroplasts with the tripeptide leucyl-glycyl-glycine. The results of these experiments are represented in table VI.

TABLE VI.

*Changes in the content of protein and non-protein nitrogen during incubation of bean chloroplasts in the presence of leucyl-glycyl-glycine and ATP* (in mg. N)

| | Incubation period (days) | |
|---|---|---|
| | 0 | 3 |
| **Controls :** | | |
| **non-protein N . . .** | 1.45 | 1.48 |
| **protein N . . . . .** | 3.21 | 3.15 |
| **Experimental :** | | |
| **non-protein N . . .** | 1.98 | 1.17 |
| **protein N . . . . .** | 3.14 | 3.47 |

It should be pointed out that the increase in protein nitrogen at the expense of peptide nitrogen is observed only in plastides isolated from chlorophyll-containing tissues of young plants. As a rule, an increase in protein nitrogen at the expense of peptide nitrogen could not be discovered in plastides isolated from the leaves of etiolated plants and of plants in the later phases of development.

The fact that chloroplasts participate in the synthesis of proteins is confirmed also by the results of experiments with labelled compounds. It has been established that labelled glycine incubated with chloroplasts in the presence of ATP, fumarate and succinate is not incorporated into their proteins.

On the basis of earlier experiments with non labelled compounds we have explored the possibility of incorporating the labelled dipeptide glycyl-glycine into the proteins of chloroplasts. Results are shown in the table VII. It was discovered that when chloroplasts are incubated as described above with $^{14}C$-glycyl-glycine the proteins of plastides acquire radioactive properties

TABLE VII.

*Incorporation of $^{14}C$-glycyl-glycine into the proteins of bean choloroplasts (in µg./mg. of proteins)*

| Age of bean | 2 weeks | 3 weeks |
|---|---|---|
| Expt. nr. 1 . . . . . . . . | 2.19 | 1.17 |
| Expt. nr. 2 . . . . . . . . | 2.35 | 1.17 |
| Expt. nr. 3 . . . . . . . . | 2.03 | 0.75 |

corresponding to 0.75-2.35 µg. of peptide mg. of the chloroplasts protein.

The labelled dipeptide is not incorporated in the proteins of the chloroplasts without added ATP.

Taking into consideration the increase in the protein nitrogen of chloroplasts in the presence of peptides, and the selective use of the labelled $^{14}C$-glycyl-glycine for the synthesis of protein, it may be assumed that the transpeptidase reaction is one of the possible pathways of protein synthesis in the green plastides.

Further investigations have shown that the chemical process involved in protein synthesis depends to a large degree on the condition of the isolated plastides. It has been found that after washing the chloroplasts of bean leaves three times with saccharose phosphate buffer in the cold, the plastides acquire the capacity of incorporation free labelled glycine into their proteins.

Moreover, it has been established that the rate of incorporation depends definitely upon the pH. The curve showing the pH-dependance of the incorporation

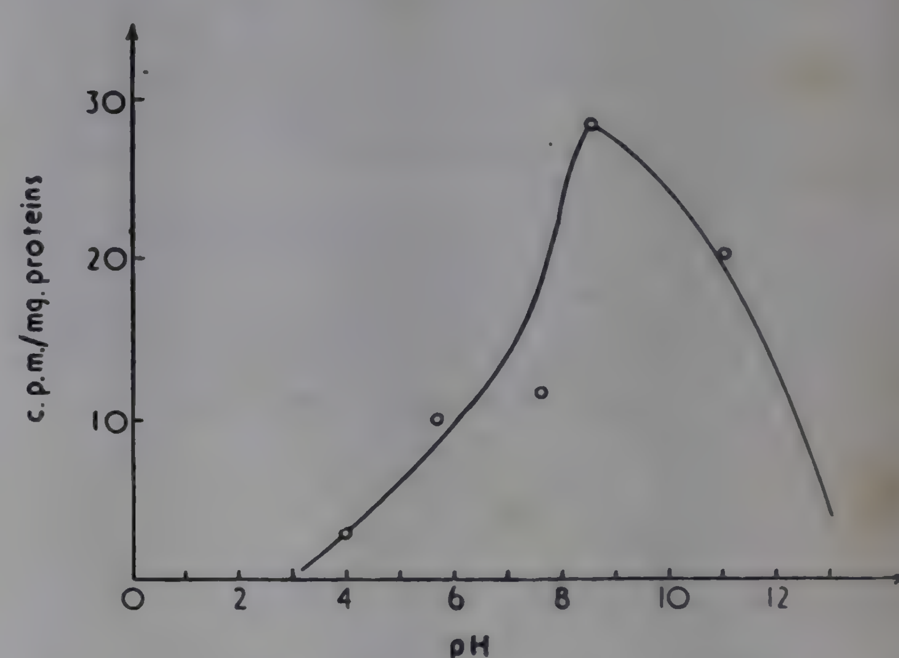


FIG. 5. — pH dependance of the incorporation of glycin-$^{14}C$ in the proteins of chloroplasts.

of labelled glycine in the proteins of chloroplasts indicates that this reaction takes place within a wide range of pH and has a maximum on the alcaline side (figure 5).

Table VIII contains some results of the experiments on the incorporation of labelled glycine in the proteins of chloroplasts.

The data of table VIII show that in the incubation of homogenate and various fractions of bean leaves with $^{14}C$-glycine, the incorporation per unit of protein in chloroplasts is 3-4 times greater than in the total homogenate.

In the same conditions the radioactivity of proteins in the supernatant fluid is very low, near the radioactivity of homogenate. Three preliminary washings of chloroplasts with a saccharose phosphate buffer increase the incorporation of $^{14}C$-glycine into their proteins fifteen to twenty fold. This effect may be completely abolished by adding the supernatant fluid to the fraction of washed chloroplasts.

TABLE VIII.

*Incorporation of $^{14}C$-glycine into the proteins of bean leaves (c.p.m./mg. proteins)*

| Expt. nr. | Total homogenate | Isolated chloroplasts | Supernatant after separation of chloroplasts | Chloroplasts washed three times with saccharose-phosphate | Washed chloroplasts + supernatant |
|---|---|---|---|---|---|
| 1 | 2.5 | 10.5 | 1.2 | 141.6 | 2.5 |
| 2 | 3.8 | 13.4 | 2.9 | 192.7 | 2.6 |
| 3 | 3.0 | 10.6 | 2.6 | 157.8 | 3.2 |

It is obvious that in the ordinary procedure, isolated chloroplasts contain substances existing or originating in the cytoplasm in the process of isolation of the chloroplasts, which supress the enzymatic incorporation of $^{14}C$-glycine into the proteins of the chloroplasts. According to the preliminary data, this inhibiting agent is a non-protein substance. It has been found out that this agent does not affect the rate of incorporation of radioactive glycine by the proteins of rat liver cell fractions composed of mitochondria and microsomes.

In this connection it should be pointed out that the synthesis of the peptide bond may proceed in the chloroplasts only because the enzymatic system of the plastides is separated from the inhibiting agents present in the cytoplasm.

## *Conclusion*

The experimental data mentioned above allow some general conclusions.

In the light of these facts the former concept on the role of chloroplasts in the vital functions of plants seems rather one-sided. There is no doubt at the present that chloroplasts not only take part in the assimilation of carbon dioxide by green plants but also play a very important part in the various biochemical transformations of the cell.

The manifold biochemical properties of plastides are due to the presence of polyvalent enzymatic systems in them. Enzymes are found in plastides in free, loosely bound and firmly bound state. The strength of the bond of enzymes with the protein complex of plastides changes in the course of development of an organism and as a result of alterations in its physiological state. The developments of the organism is also attented by the qualitative and quantitative changes of ribonucleic acid and in the amount of proteins in chloroplasts.

The synthesis and oxidation of fatty acids of the lipoid fraction and $^{32}P$ incorporation into phospholipids are observed in the chloroplasts. Under certain experimental conditions, it is possible to show the increase in protein nitrogen in chloroplasts at the expense of non-protein nitrogen, as well as the incorporation of the $^{14}C$-glycine and $^{14}C$-glycyl-glycine into the proteins. The rate of incorporation depends on the nature of the added compound and on the physiological state of the chloroplasts.

Thus, the whole body of experimental evidence and observation gives us ground for the belief that plastides play a very important part in the biochemical functions of the plant cell.

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# БИОХИМИЧЕСКИЕ СВОЙСТВА ПЛАСТИД

по Н. М. СИСАКЯН

Исследования биохимических свойств протоплазменных структур, осуществленные за последнее десятилетие, привели к установлению ряда принципиально важных положений. Оказалось, что многие энзиматические реакции в живой клетке связаны с элементами протоплазменных структур. Для растительной клетки наиболее изученными структурными образованиями являются хлоропласты, дифференциация и становление которых определяют специфику растения.

Примерно 10—15 лет назад в физиологии и биохимии растений еще господствовало представление, согласно которому роль хлоропластов в растительной клетке ограничивалась лишь их участием в процессах фотосинтеза. Изучение разнообразных биохимических свойств пластид стало возможным только после широкого использования в экспериментальной технике метода дифференциального центрифугирования, применяемого для разделения протоплазменных структур.

Всестороннее изучение биохимических свойств пластид показало, что в них локализованы многие энзиматические системы клетки.

В своем докладе я хотел бы остановиться на некоторых наиболее важных итогах работ по изучению биохимической активности пластид, осуществляемых в нашей лаборатории. Наши исследования, проведенные совместно с А. М. Кобяковой, И. И. Филиппович, М. С. Одинцовой, Б. П. Смирновым и И. М. Мосоловой, показали, что энзиматическая активность пластид зависит как от природы и физиологического состояния организма, так и от характера связи энзимов с протеидным комплексом пластид.

### *О зависимости энзиматической активности пластид от особенностей организма*

Наши исследования показали, что активность энзимов в пластидах зависит от степени устойчивости данного растения к хранению. Оказалось, что активность липооксидазы в хромопластах двух разных сортов моркови при одинаковых условиях хранения различная (табл. 1).

Таблица 1

*Активность липооксидазы в хромопластах моркови*

(в μл $O_2$ на 1 г сухого вещества за 15 минут)

| Дата опыта | Сорт | |
|---|---|---|
| | нантская | московская зимняя ОА 515 |
| 15 ноября | 276 | 0 |
| 7 декабря | 227 | 0 |
| 12 февраля | 0 | 0 |
| 5 апреля | — | 182 |
| 10 мая | — | 435 |

В хромопластах, изолированных из сорта моркови, устойчивого к длительному хранению (московская зимняя ОА 515), в начале хранения и в процессе хранения не удается обнаружить липооксидазной активности до февраля включительно. Активность энзима обнаруживается в заметной степени в апреле и резко нарастает в мае. У сорта, неустойчивого к длительному хранению (нантская), активность липооксидазы в хромопластах обнаруживается уже в начале хранения, затем она постепенно уменьшается, снижаясь к концу хранения (февраль) до нуля.

Необходимо указать, что при тех же условиях опыта в хромопластах устойчивого к хранению сорта моркови после автолиза обнаруживается значительная липооксидазная активность, что, повидимому, обусловливается изменением связи энзима с протеидным комплексом хромопластов.

### *О характере связи энзимов с протеидным комплексом пластид*

Дальнейшие исследования показали, что большинство энзимов в различных типах пластид связано со структурой пластид, причем тип связи меняется в процессе онтогенетического развития организма и под влиянием различных воздействий. Нарушение покоя и этиолирование растений приводят к лабилизации связи энзима с протеидным комплексом пластид.

В результате этиолирования или даже кратковременного выдерживания зеленых растений в темноте в течение нескольких дней наблюдается значительное повышение количества свободных и непрочно связанных энзимов. Фотосинтез же, напротив, способствует стабилизации этой связи.

Связь энзимов с протеидным комплексом пластид можно совсем разрушить созданием высокой осмотической концентрации, продолжительным автолизом, обезвоживанием, замораживанием, действием различных растворителей и другими средствами.

Как было установлено в наших исследованиях, в процессе хранения органов отложения запасных веществ растения также наблюдается лабилизация связи энзима со структурами пластид.

Таблица 2

*Изменение прочности связи инвертазы с протеидным комплексом пластид*

| Характер связи | Количество инвертазы, % (по активности) | |
|---|---|---|
| | сентябрь | декабрь |
| Свободная | 14.28 | 33.58 |
| Непрочно связанная | 32.25 | 43.54 |
| Прочно связанная | 53.35 | 32.88 |

В табл. 2 показано изменение типа связи инвертазы в лейкопластах сахарной свеклы после четырехмесячного хранения (декабрь) по сравнению с лейкопластами, изолированными в начале хранения (сентябрь).

Как видно из приведенных данных, уже после четырехмесячного хранения весьма существенно меняется характер связи инвертазы в сторону увеличения количества свободного и непрочно связанного энзима за счет уменьшения прочно связанного.

Дальнейшие исследования показали, что способность белков образовывать комплексы с липоидами, нуклеиновыми кислотами и в целом с протеидным комплексом пластид во многом обусловливается теми же самыми факторами, которые влияют на изменение связи с этим комплексом различных энзимов. Так, в процессе старения происходит изменение количества связанных липоидов, меняется количественный и качественный состав рибонуклеиновой кислоты, белковых соединений в пластидах и одновременно с этим изменяется прочность связи различных энзимов с протеидным комплексом пластид. Поэтому естественно допустить, что причины изменения прочности связи энзимов со структурами пластид следует искать в изменениях общей комплексообразующей способности протеидов пластид.

Возможность лабилизации связи энзимов со структурными элементами пластид под влиянием различных условий, как нам представляется, необходимо учитывать при оценке энзиматической активности клеточных структур. Поэтому едва ли можно согласиться с мнением тех исследователей, которые свои выводы об энзиматических свойствах отдельных элементов протоплазменных структур, например хлоропластов, строят на результатах опытов, проведенных без учета этого обстоятельства. Следует также учитывать, что при лабилизации связи

энзимов со структурами может происходить не только переход энзимов в раствор, но также и их межструктурный обмен.

Наличие в пластидах многих энзиматических систем, сравнительно высокое содержание в них белков и нуклеиновых кислот свидетельствуют о том, что эти структуры играют исключительно важную роль в разнообразных биохимических функциях клетки, в том числе и в синтетических процессах.

*О циклофоразной системе хлоропластов*

Большой интерес представляет исследование энзимов циклофоразной системы в растениях. В литературе имеются указания на большую лабильность циклофоразной системы в животной клетке. Многие неудачи при попытке обнаружения этой системы в растительных тканях возможно объясняются ее лабильностью и в растениях, хотя отдельные звенья этой каталитической системы, так же как и кислоты трикарбоксильного цикла, уже обнаружены в различных растениях.

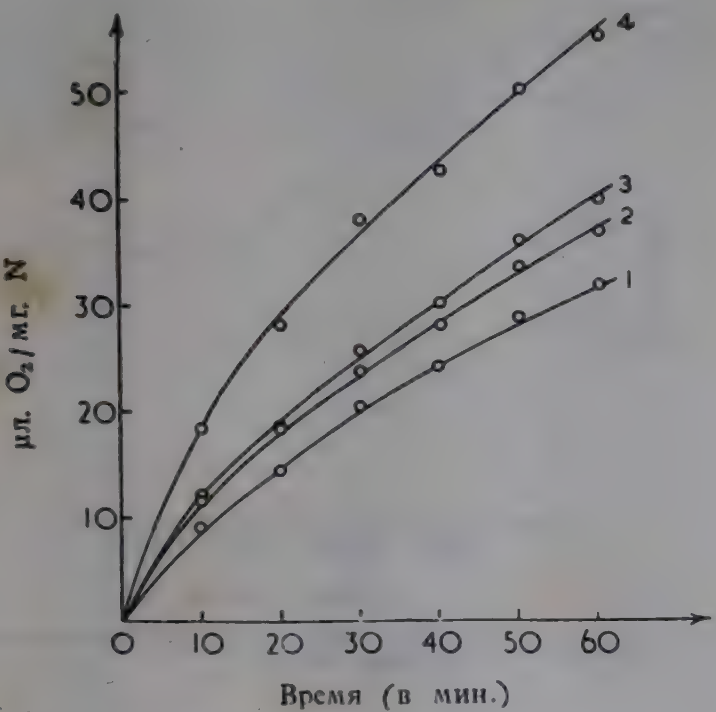


Рис. 1. — Поглощение кислорода изолированными хлоропластами фасоли: (1) хлоропласты, (2) хлоропласты + сукцинат, (3) хлоропласты + цитрат, (4) хлоропласты + фумарат.

В своей работе по исследованию циклофоразной системы в изолированных хлоропластах мы столкнулись с серьезными трудностями, многие из которых еще не преодолены.

Тем не менее нам удалось показать, что в определенных условиях опыта хлоропласты, изолированные из осенних листьев сахарной свеклы и из листьев фасоли, окисляют некоторые кислоты цикла Кребса. На рис. 1 показаны результаты опыта с хлоропластами фасоли, из которых видно, что фумаровая, лимонная и янтарная кислоты окисляются изолированными хлоропластами. Как эти, так и имеющиеся уже данные еще недостаточны для окончательного суждения о распространенности циклофоразной системы в растениях и о ее локализации и роли в определенных структурных образованиях растительной клетки.

Исследование роли хлоропластов в синтетических функциях растительной клетки в нашей лаборатории проводилось в трех различных направлениях: — изучение синтеза жирных кислот и включения фосфора в фосфолипиды хлоропластов; — определение зависимости между количеством рибонуклеиновой кислоты и количеством белка в хлоропластах; — изучение синтеза пептидной связи в изолированных хлоропластах.

*О синтезе и окислении жирных кислот и включении фосфора в фосфолипиды хлоропластов*

Химизм синтеза и превращения жирных кислот, хорошо изученный в животном организме, почти не исследован в растительных тканях. Не имеется достаточных сведений также и о роли структурных образований растительной клетки в процессах превращения жирных кислот.

Работы нашей лаборатории показали, что в липоидной фракции изолированных хлоропластов высшие жирные кислоты составляют примерно 55-60 % в расчете на сухой вес. Оказалось, что хлоропласты обладают ярко выраженной способностью к окислению как ненасыщенных, так и насыщенных жирных кислот, таких, как линолевая, линоленовая, олеиновая и пальмитиновая.

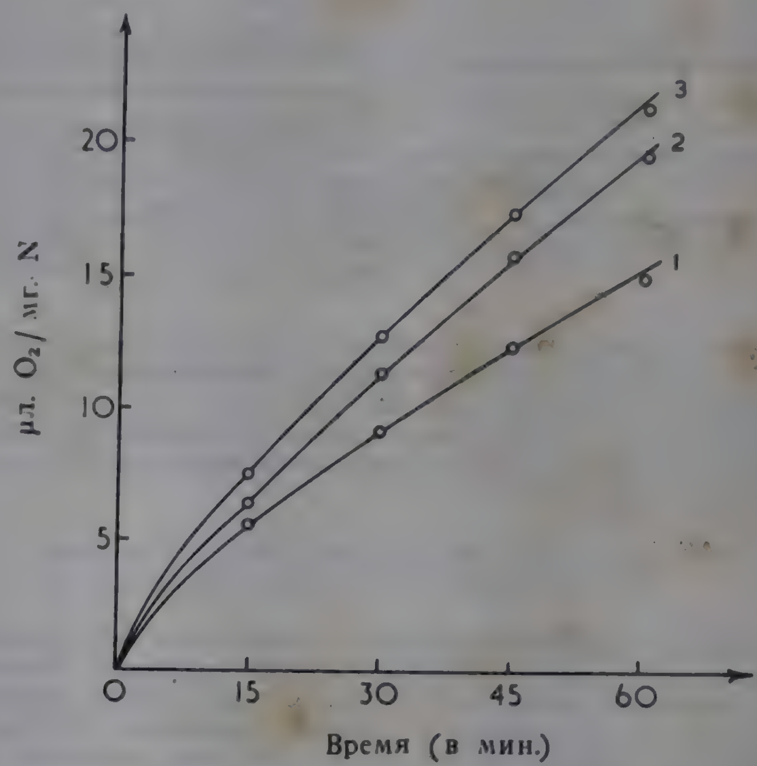


Рис. 2. — Скорость окисления жирных кислот изолированными хлоропластами: (1) пальмитиновая кислота, (2) олеиновая кислота, (3) линолевая + линоленовая кислоты.

На рис. 2 приведены результаты опытов по окислению жирных кислот хлоропластами листьев фасоли, из которых видно, что хлоропласты не только обладают липооксидазным действием, связанным с окислением линолевой и линоленовой кислот, но содержат также ферментативную систему, обусловливающую более глубокое окисление таких жирных кислот, как олеиновая и пальмитиновая. Химизм этого явления остается пока не выясненным. Наши попытки стимулировать эти реакции добавлением ДПН и АТФ не увенчались успехом, тогда как известно, что аналогичные опыты с растительными митохондриями показали важность этих соединений для окислительных процессов.

Дальнейшие исследования показали, что при инкубации хлоропластов, изолированных из семядоли десятидневных проростков подсолнечника, с радиоактивным ацетатом калия происходит включение ацетата в состав высших жирных кислот липоидной фракции (табл. 3).

Таблица 3

*Включение ацетата в высшие жирные кислоты хлоропластов*

| Номер опыта | На 100 мг жирных кислот | |
|---|---|---|
| | активность в имп/мин. | включено ацетата в μг |
| 1-й . . . . . . . . . . | 1075 | 26.2 |
| 2-й . . . . . . . . . . | 1340 | 21.4 |

Прибавление АТФ не влияет на процесс включения ацетата. Следует указать, что хлоропласты, изолированные из листьев фасоли, не обнаружили способности включать ацетат в состав своих жирных кислот. На основании полученных результатов нельзя объяснить, чем обусловливается наблюдающееся различие. Возможно, что в хлоропластах, изолированных из листьев фасоли, система, катализирующая включение ацетата в состав жирных кислот, является гораздо более лабильной, чем та же система в хлоропластах из семядоли проростков

подсолнечника, однако не исключена возможность, что такое различие обусловлено спецификой обмена веществ у названных растений.

Было установлено также, что при инкубации хлоропластов с радиоактивным фосфором происходит включение фосфора в фосфолипиды (табл. 4).

Таблица 4

*Включение радиоактивного фосфора в фосфолипиды хлоропластов фасоли*

| Номер опыта | На 10 мг фосфолипидов | |
|---|---|---|
| | активность в имп/мин. | включено $^{32}P$ в µг |
| 1-й | 5590 | 4.28 |
| 2-й | 7910 | 6.08 |

В табл. 4 приведены результаты опытов по включению фосфора в фосфолипиды хлоропластов, изолированных из десятидневных проростков фасоли. Оказалось, что этот процесс осуществляется лишь в присутствии адениловой кислоты, что наводит на мысль о возможности включения фосфора в фосфолипиды через АТФ.

### *О рибонуклеиновой кислоте (РНК) и ее связи с количеством белков в хлоропластах*

В наших исследованиях было обнаружено, что пластиды, выделенные из листьев сахарной свеклы, шпината, подсолнечника, табака, а также из корней сахарной свеклы и моркови, содержат рибонуклеиновую кислоту, количество которой колеблется в зависимости от вида и возраста растений от 0.5 до 3.5 % расчете на сухой вес. Оказалось, что в процессе развития растительного организма количество РНК в хлоропластах, так же как и в других биологических объектах, подвергается закономерным изменениям. В хлоропластах молодых листьев РНК содержится примерно в 2—3 раза больше, чем в хлоропластах, полученных из старых листьев. Характерно, что при этом меняется и качественный состав РНК (табл. 5).

Таблица 5

*Содержание азотистых оснований в РНК хлоропластов листьев табака в период бутонизации*

| Листья ярусов | Гуанин | Аденин | Пурины/пиримидины |
|---|---|---|---|
| Верхний | 1.08 | 1 | 1.64 |
| Средний | 1.22 | 1 | 1.30 |
| Нижний | 1.31 | 1 | 1.27 |

Как видно из табл. 5, в РНК, извлеченной из хлоропластов табака холодной 10 %-ной хлорной кислотой, по мере старения листьев увеличивается отношение гуанина к аденину и уменьшается отношение пуринов к пиримидинам, т. е. РНК как бы обогащается пиримидиновыми основаниями. Явление обогащения РНК пиримидиновыми основаниями было нами обнаружено также и в хлоропластах сахарной свеклы по мере старения растения. Следует также отметить, что отношение азотистых оснований в РНК хлоропластов не является эквимолекулярным. Повидимому, это обусловлено тем, что РНК хлоропластов не имеет тетрануклеотидного строения.

Значительный интерес представляет наблюдающийся в процессе развития организма параллелизм изменения количества РНК и белка в хлоропластах. Как это видно из рис. 3, по мере старения одновременно с уменьшением количества РНК происходит падение содержания белка в хлоропластах листьев сахарной свеклы.

Интересно отметить, что уменьшение количества РНК сопровождается повышением активности рибонуклеазы в хлоропластах. Так, например, при содержании РНК в хлоропластах листьев сахарной свеклы 4-й розетки, равном 0.8 %, активность рибонуклеазы составляет 5.91 мг фосфора на 100 мг азота. В этих же условиях опыта в хлоропластах листьев 12-й розетки при увеличении содержания РНК до 2.1 % активность рибонуклеазы снижается до 2.14 мг фосфора на 100 мг азота.

Наблюдаемые факты, а также широко распространенное мнение об участии нуклеиновых кислот в синтезе белка дают основание предполагать, что в хлоропластах происходит синтез пептидной связи.

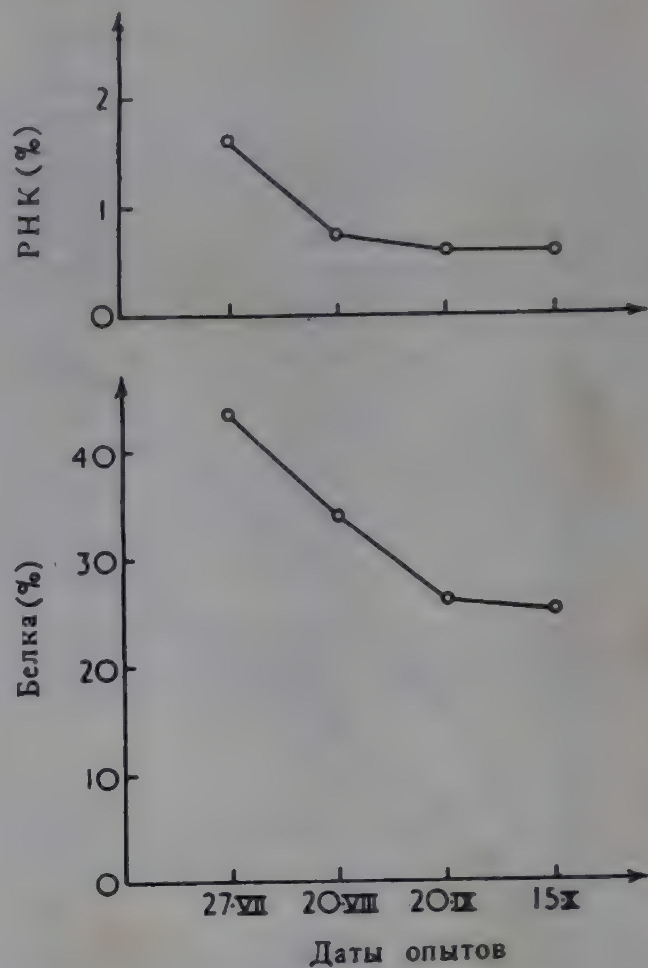


Рис. 3. — Изменение количества РНК и белка в хлоропластах в процессе развития растения.

### *О синтезе белка в хлоропластах*

Положение о возможной роли хлоропластов в синтезе пептидной связи нашло экспериментальное подтверждение в серии опытов, проведенных в нашей лаборатории. Было показано, что при инкубации хлоропластов клевера в присутствии смеси аминокислот (состоящей из гликокола, треонина, серина, тирозина, валина, аланина, аргинина, лизина, аспарагиновой и глютаминовой кислот) в соотношениях, соответствующих таковым в белках пластид, не происходит какого-либо заметного изменения количества белкового азота. Однако в отсутствии аминокислот наблюдается прирост белкового азота за счет небелкового, составляющий 6 %, причем убыли свободных аминокислот, определяемых нингидринным методом, при этом не обнаруживается. Этот факт наводит на мысль, что наблюдаемый прирост белкового азота, возможно, происходит за счет свободных пептидов.

Дальнейшие опыты показали, что в действительности в хлоропластах в присутствии добавленных ди- и трипептидов наблюдается нарастание количества белкового азота. Как это видно из рис. 4, инкубация хлоропластов сахарной свеклы с глицил-глицином в присутствии АТФ, сукцината и фумарата калия приводит к увеличению белкового азота и уменьшению небелкового. Прирост белкового азота составляет примерно 12 % от общего азота хлоропластов.

Подобные изменения в соотношениях между белковым и небелковым азотом можно наблюдать только в присутствии АТФ. Без добавления АТФ такую закономерность обнаружить не удается.

Подобное же явление, выраженное в меньшей степени, было обнаружено при инкубации хлоропластов с трипептидом (лейцил-глицил-глицином). Результаты этих опытов представлены в табл. 6.

Таблица 6

*Изменение содержания белкового и небелкового азота при инкубации пластид фасоли с лейцил-глицил-глицином и АТФ (в мг азота)*

| Время инкубации (в сутках) | Контроль | | Опыт | |
|---|---|---|---|---|
| | небелковый азот | белковый азот | небелковый азот | белковый азот |
| 0 | 1.45 | 3.21 | 1.98 | 3.14 |
| 3 | 1.48 | 3.15 | 1.57 | 3.47 |

Следует указать, что нарастание белкового азота за счет азота пептидов наблюдается только в пластидах, выделенных из хлорофиллоносных тканей молодых растений. В пластидах, изолированных из листьев этиолированных растений, а также растений поздних фаз развития прироста белкового азота за счет азота пептидов, как правило, обнаружить не удается.

Об участии хлоропластов в белковом синтезе клетки свидетельствуют также данные опытов с мечеными соединениями. Было установлено, что меченый глицин $^{14}C$ при инкубации с хлоропластами в присутствии АТФ, фумарата и сукцината калия не включается в состав их белков.

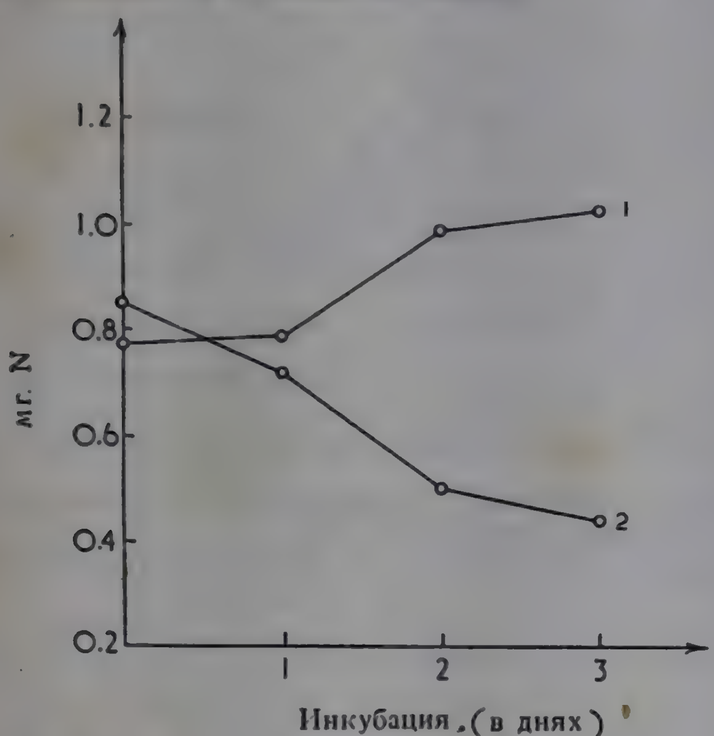


Рис. 4. — Изменение содержания белкового и небелкового азота при инкубации хлоропластов с дипептидом и АТФ: (1) белковый азот, (2) небелковый азот.

Исходя из результатов предыдущих опытов с немечеными соединениями, мы провели исследование по выяснению возможности включения в белки хлоропластов меченого дипептида — глицил-глицина $^{14}C$. Оказалось, что при инкубации хлоропластов в условиях опытов, описанных выше с меченым глицил-глицином $^{14}C$, белки пластид приобретают радиоактивность, соответствующую 0.75—2.35 $\mu$г пептида на 1 мг белка хлоропластов.

Без добавления АТФ включение меченого дипептида в белки хлоропластов не происходит (табл. 7).

Таблица 7

*Включение глицил-глицина $^{14}C$ в белки хлоропластов фасоли*

| Возраст фасоли | Номер опыта | $\mu$г на мг белка |
|---|---|---|
| 2 недели . . . . . . . . | 1-й | 2.19 |
| | 2-й | 2.35 |
| | 3-й | 2.03 |
| 3 недели . . . . . . . . | 1-й | 1.17 |
| | 2-й | 1.17 |
| | 3-й | 0.75 |

Исходя из увеличения белкового азота хлоропластов в присутствии пептидов, а также избирательного использования для синтеза белка меченого дипептида глицил-глицина $^{14}C$, можно допустить, что одним из возможных путей синтеза белка в зеленых пластидах является реакция транспептидазного типа.

Дальнейшие исследования показали, что химизм синтеза белка в значительной степени зависит от состояния изолированных пластид. Оказалось, что после трехкратного промывания хлоропластов листьев фасоли сахарозо-фосфатным буфером на холоду пластиды приобретают способность включать в свои белки свободный меченый глицин $^{14}C$.

При этом было установлено, что интенсивность включения определенным образом зависит от pH среды. Кривая зависимости включения радиоактивного глицина в белки хлоропластов от величины pH показывает, что эта реакция осуществляется в широких пределах изменения pH и имеет максимум, сдвинутый в щелочную сторону (рис. 5).

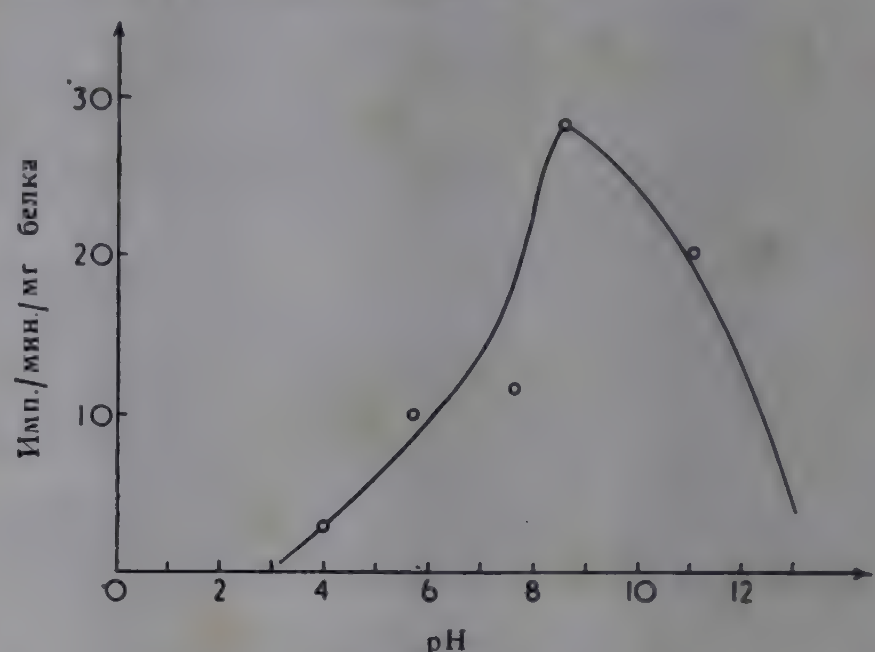


Рис. 5. — Зависимость включения глицина $^{14}C$ в белки хлоропластов от pH среды.

В табл. 8 приведены некоторые результаты опытов по включению радиоактивного глицина в белки хлоропластов.

Цифры табл. 8 показывают, что при инкубации гомогената и различных фракций листьев фасоли с меченым глицином $^{14}C$ включение изотопа на единицу белка в хлоропластах в 3—4 раза выше, чем в гомогенате.

Таблица 8

*Включение глицина $^{14}C$ в белки фракций листьев фасоли в имп-мин. на 1 мг белка*

| Номер опыта | Гомогенат | Хлоропласты | Центрифугат после осаждения хлоропластов | Хлоропласты, промытые три раза сахарозо-фосфатным раствором | Хлоропласты, промытые три раза + центрифугат |
|---|---|---|---|---|---|
| 1-й | 2.5 | 10.5 | 1.2 | 141.6 | 2.5 |
| 2-й | 3.8 | 13.4 | 2.9 | 192.7 | 2.6 |
| 3-й | 3.0 | 10.6 | 2.6 | 175.8 | 3.2 |

В этих же условиях радиоактивность белков центрифугата очень низка и приближается к радиоактивности гомогената. Трехкратное предварительное промывание хлоропластов сахарозо-фосфатным буфером приводит к увеличению включения изотопа в их белки в 15—20 раз. Этот эффект полностью снимается добавлением к фракции промытых хлоропластов центрифугата.

Очевидно, в обычных условиях изолирования хлоропласты содержат примеси веществ, имеющихся или возникающих в цитоплазме в ходе выделения хлоропластов, которые подавляют энзиматическое включение глицина в белки хлоропластов. Согласно предварительным данным этот ингибирующий фактор

является веществом небелковой природы. Оказалось, что этот фактор не влияет на интенсивность включения радиоактивного глицина в белки клеточной фракции печени крысы, состоящей из митохондрий и микросом.

В связи с этим следует отметить, что в хлоропластах синтез пептидной связи осуществляется, повидимому, благодаря пространственной разобщенности энзиматических систем пластид и ингибирующих веществ цитоплазмы.

Полученные нами экспериментальные данные позволяют придти к некоторым общим выводам.

В свете этих фактов прежние представления о значении хлоропластов в жизнедеятельности растений кажутся несколько односторонними. В настоящее время не подлежит сомнению, что хлоропласты принимают участие не только в процессах ассимиляции $CO_2$ зелеными растениями, но и играют исключительно важную роль в разнообразных биохимических превращениях клетки.

Многогранность биохимических свойств пластид обусловлена наличием в них поливалентных энзиматических систем. Энзимы находятся в пластидах в свободном, непрочно связанном и прочно связанном состоянии. Характер связи энзимов с протеидным комплексом пластид меняется в процессе развития организма и при изменении его физиологического состояния. По мере развития организма меняется также качественный и количественный состав рибонуклеиновой кислоты и количество белка хлоропластов.

В хлоропластах наблюдается синтез и окисление жирных кислот липоидной фракции и включение фосфора в фосфолипиды. В определенных условиях опыта удается показать нарастание белкового азота в хлоропластах за счет уменьшения небелкового, а также включение меченого глицина $^{14}C$ и глицил-глицина $^{14}C$ в состав белков хлоропластов. Интенсивность включения зависит как от природы добавляемого соединения, так и от физиологического состояния хлоропластов.

Таким образом, совокупность экспериментальных фактов и наблюдений позволяет считать, что пластиды играют весьма существенную роль в биохимической функции растительной клетки.

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# Biochemical disorders in peripheral neuropathies (*)

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(*Received 24 September 1955*)

Degenerative changes commencing at the distal ends both of the peripheral nerves and of the long fibre tracts in the spinal cord, and progressing upwards in the direction of the cell body from which the fibre is derived, are well recognised in a variety of different conditions. Examples are provided by the polyneuritis that can accompany thiamine deficiency, the subacute combined degeneration of the spinal cord, usually associated also with a peripheral neuritis, that occurs in certain cases of vitamin $B_{12}$ deficiency, and the motor paralysis caused by intoxication with tri-*o*-cresyl phosphate. Indeed when the relative dimensions of the nerve cell body and the axon supplying, say, a group of muscle fibres in the foot of an adult man are considered, it might be expected that a tissue showing such specialised features, both anatomical and physiological, would also exhibit some specialised aspects of metabolism, derangements of which might readily occur; and such indeed appears to be the case. This high degree of anatomical specialisation in the nervous system is however a barrier in the way of advance, and our knowledge of the detailed biochemistry of different parts of the neurone, or even of the different types of neurone or glial cell, or indeed even of localised areas of the central nervous system, is still scanty.

It has of course for long been known that cerebral grey matter has a higher rate of oxygen utilization in the presence of glucose than has white matter, while peripheral nerve shows a lower rate still. This high metabolic rate of grey matter is not, however, due solely to the cell bodies, but is also apparently associated with the dendrites. Thus, Holmes in 1932 (1) found that the trigeminal ganglion, a structure containing cell bodies but no dendrites or synapses, has a low respiratory rate. More recently Dixon (2) studied the rates of glycolysis of superficial and deep slices of rabbit cerebral cortex; the superficial slices, consisting mainly of fibres and dendrites with few cell bodies, were found to glycolyse as high as or even higher than the deep slices containing predominantly cell bodies. If this high metabolic rate of the dendrites is found to be true also for the terminal parts of the long axons, this would certainly be a development of real importance in our understanding of the early stages of the peripheral neuropathies.

Studies on peripheral nerves undergoing Wallerian degeneration after transection are also yielding important information concerning the localisation and activity of various enzyme systems during the processes of nerve degeneration and demyelination, and during the later stages of regeneration and repair. Rossiter and his colleagues in Canada have recently contributed much valuable information in this field; they have, for example, been able to conclude that the alkaline phosphatase of peripheral nerve is associated with the axon rather than with the nerve sheath, whereas the acid phosphatase is very largely a component of the Schwann cells (3). There is now also good evidence, obtained by both histochemical and standard biochemical techniques, that the cholinesterases are also located differently in the nervous system, the true cholinesterase being situated in the axon and particularly in the region of the synapses, while the pseudo-cholinesterase is situated in the Schwann cells of the peripheral nerves and in the glial cells of the central nervous system (4, 5). But important though these studies are, particularly in connection with the primary demyelinating diseases of the central nervous system, I want to turn from the anatomical to the more directly functional aspects of the biochemistry of nerve cells, since it is along these lines that the advances of greatest practical significance at the present time have been made.

From the standpoint of the chemical pathologist, the polyneuritis of thiamine deficiency occupies the central position of this group of conditions, since it was from the work of Sir Rudolph Peters on avian polyneuritis in rice-fed pigeons that our present knowledge of the biochemical lesion underlying thiamine deficiency developed (see 6). And with this came also the conception that other biochemical derangements, either related to that occurring in thiamine deficiency or of a fundamentally different kind, may underlie other types of polyneuritis.

In certain instances we have gone some distance towards an understanding of these derangements in different

(*) Lecture given on Tuesday 2 August 1955 at a joint session of section 14 (chemical pathology and immunochemistry) and section 16 (clinical chemistry). The programme of these sections had been decided in collaboration with the International Association of Clinical Chemists.

types of peripheral neuropathies. In arsenical neuritis, for example, it is believed that inactivation of one of the components of the pyruvate and α-ketoglutarate oxidase systems of enzymes occurs by reaction of the arsenical with an essential thiol grouping (7, 8). This arsenic-sensitive component may possibly be related to the dithiol lipoic acid or the lipothiamide co-enzyme factor (see 9) since for adequate protection of pyruvate metabolism in an arsenic-intoxicated animal a dithiol such as 2,3-dimercaptopropanol (British Anti-Lewisite, or BAL) is effective, whereas monothiols are not (10). There are reasons also for thinking that the polyneuritis caused by certain other metal poisons, antimony, mercury or copper, for example, may also be due to a similar biochemical disorder.

In addition to these metal poisons, it is known both from clinical and experimental observations that certain organic compounds can also inhibit pyruvate oxidation. Thus, ingestion of the toxic alkaloid sanguinarine obtained from argemone oil, has been shown to be the probable cause of the epidemic dropsy resembling wet beri-beri that occurs in certain parts of India. And, as has been shown by Sarkar (11), this compound is a potent inhibitor of pyruvate oxidation *in vivo*. The blood pyruvate levels of rats poisoned with this alkaloid are significantly raised, and, again, protection can be brought about by treatment with 2,3-dimercaptopropanol.

It is well known also that anoxia and liver damage can cause an accumulation of pyruvate. It is clear therefore that the finding of a high blood pyruvate level would not, by itself, indicate that a case of chronic peripheral neuritis is due to a state of thiamine deficiency.

### *Blood pyruvate levels in polyneuritis*

At Guy's Hospital, in London, we have recently determined (12) the blood pyruvate levels in a series of over 50 unselected cases of polyneuritis, after administering a loading dose of glucose by mouth according to the technique adopted by Williams, Mason, Power and Wilder (13). We first determined the response to the administered glucose in a series of 50 controls consisting of healthy subjects and patients in whom there was no reason to suspect a thiamine deficiency or any other impairment of carbohydrate metabolism. It will be seen (table I) that in agreement with the earlier findings of Bueding, Stein and Wortis (14) and Williams and colleagues (13) only a slight rise in the blood pyruvate level occurs in normal subjects at either 60 or 90 minutes after the dosing with glucose.

TABLE I

*Blood pyruvate levels after administration of glucose derived from 50 control subjects (mg. pyruvic acid/100 ml. blood)*

| | 0 min. | 60 min. | 90 min. |
|---|---|---|---|
| Mean | 0.76 | 0.92 | 0.94 |
| S. D. (*) | 0.18 | 0.17 | 0.20 |
| Mean + 2 S. D. | 1.12 | 1.26 | 1.34 |

(*) S. D. = standard deviation.

Figure 1 summarises the results in the first 50 unselected cases of polyneuritis of various types. The values

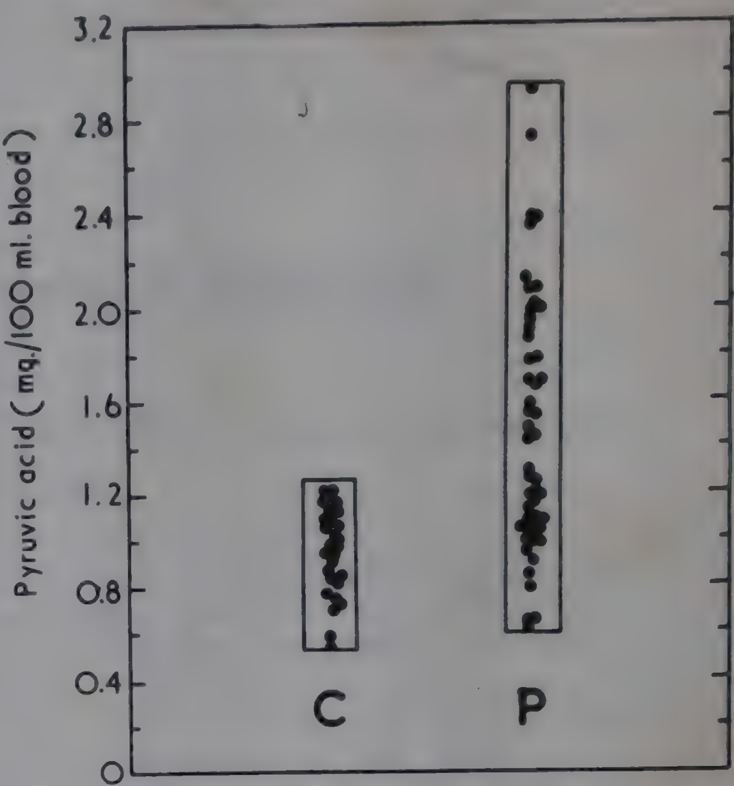


FIG. 1. — Maximal blood pyruvate values in polyneuritis (after administration of glucose). C = controls; P = polyneuritis.

charted represent the maximum value obtained at either 60 or 90 minutes after the glucose. It will be seen that some, but not all, of these cases showed a very striking elevation of the blood pyruvate level over the control group. In this series just over half the cases studied gave high values, although only three among the entire series showed high fasting values.

It was unlikely from the dietary histories of these 50 patients that approximately half of them would have been suffering from a degree of thiamine deficiency sufficiently severe to cause a significant elevation of the blood pyruvate level. Further, it is well known clinically that thiamine therapy, even when given parenterally in large doses, is only of value in a relatively small proportion of cases of peripheral neuritis. So that we are faced with the question as to the nature of the underlying abnormality responsible for these raised pyruvate levels in so many of these cases.

Polyneuritis due to a simple deficiency of thiamine can in most cases be differentiated from that due to some other cause by giving massive thiamine therapy (100 mg./day) parenterally for 14 days, and then repeating the estimation of the blood pyruvate level after a loading dose of glucose.

If the high pyruvate values found originally had been due to a lack of thiamine, it is reasonable to expect that they would have been restored to normal by 14 days of such therapy. Figure 2 shows the results of such tests carried out before and after thiamine therapy on a group of cases of polyneuritis all showing high pyruvate levels before treatment. It will be seen that in some of these cases thiamine therapy has caused the originally high pyruvate level to return to well within the normal range, whereas in others the blood

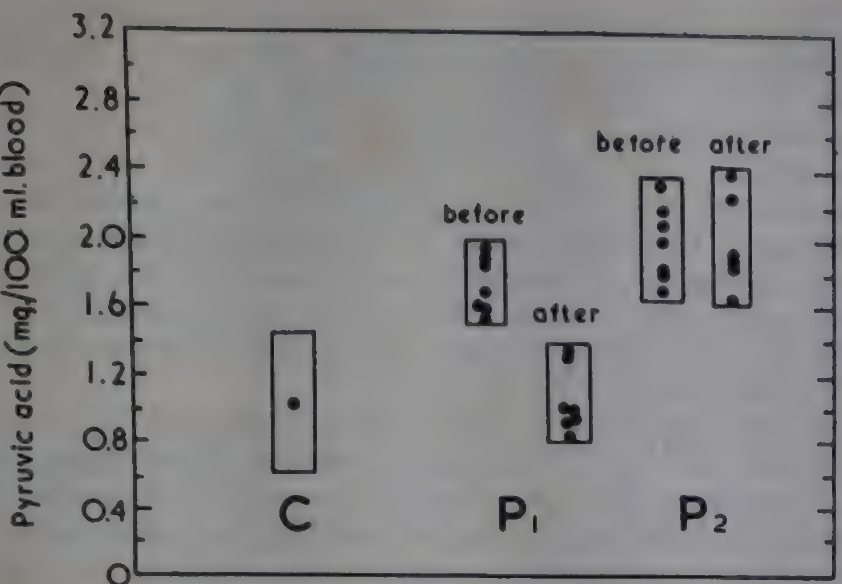


FIG. 2. — Effect of vitamin $B_1$ therapy on cases of polyneuritis with raised blood pyruvate levels (after glucose load). C = controls; $P_1$ and $P_2$ = polyneuritis.

levels have been entirely unaffected by this treatment.

From the biochemical point of view, therefore, the cases of polyneuritis which we have studied fall into three groups :

(*a*) cases in which no abnormality of pyruvate metabolism can be detected. These cases correspond to approximately one half of the total number of cases which we have studied;

(*b*) cases showing impaired pyruvate metabolism, but in whom the blood pyruvate level falls to normal after 14 days of intramuscular thiamine therapy. The polyneuritis in these cases is presumably associated with a thiamine deficiency.

(*c*) cases showing impaired pyruvate metabolism in whom this biochemical disturbance remains unaffected by massive and prolonged thiamine therapy; in these cases the polyneuritis and the impairment of pyruvate metabolism are presumably not due to a simple deficiency of thiamine, but must be due to some other cause, possibly some interference with sensitive thiol groups.

### *Subacute combined degeneration of the cord*

In connection with this last possibility some interesting speculations as to the underlying change in subacute combined degeneration of the cord are now presenting themselves as a result of recent work on the biochemical changes in vitamin $B_{12}$-deficient animals.

Although patients with subacute combined degeneration of the cord that are receiving adequate treatment with vitamin $B_{12}$ or with liver extracts show normal levels of pyruvate in the blood, we have recently had the opportunity to study three untreated cases of this condition. Not only did we find that the blood pyruvate level was high in each of these three cases, but we were able to show that treatment, limited to the parenteral administration of vitamin $B_{12}$, led to a rapid return to normal of these levels (15).

As this finding was, at the time, a surprise to us, and since the estimations of pyruvic acid had been carried out by the 2,4-dinitrophenylhydrazone method of Friedemann and Haugen (16), which also estimates to some extent other keto-acids in addition to pyruvic acid, we thought it advisable to identify more exactly this keto-acid which is present in increased amounts in the blood of these patients. More particularly did this seem necessary in view of the reports of the excretion of *p*-hydroxyphenylpyruvic acid in large quantities in the urine of patients with pernicious anaemia. We therefore carried out a separation, by paper chromatography, of the 2,4-dinitrophenylhydrazones of the keto-acids present in the blood by a modification of the technique described by Cavallini, Frontali and Toschi (17). Under our conditions we regularly find the presence of 3 'spots' in chromatograms of the keto-acid hydrazones extractable from human blood (18). The most slowly moving spot is the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid. The pyruvate 2,4-dinitrophenylhydrazone gives rise to the other two spots. It has recently been suggested by Stewart (19) and by Isherwood and Cruickshank (20) that the pyruvate 2,4-dinitrophenylhydrazone gives rise to two spots as a result of *cis-trans* isomerism round the N of the hydrazone linkage (figure 3).

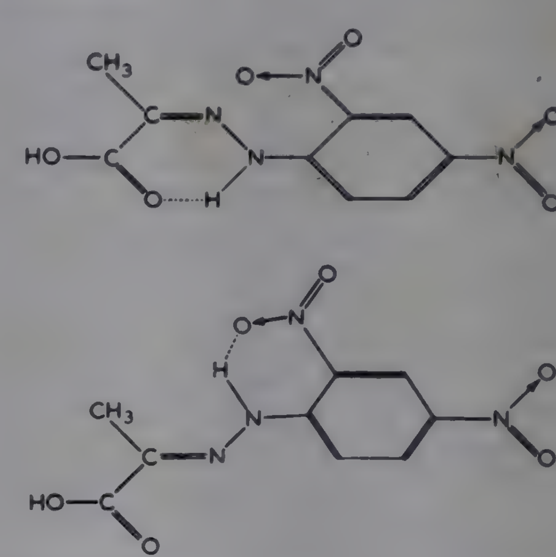

FIG. 3. — *Cis-trans* isomerism of 2, 4-dinitrophenylhydrazone of pyruvic acid.

The existence of two pyruvate spots was first shown in chromatograms of the 2,4-dinitrophenylhydrazone formed from pure sodium pyruvate. El Hawary and I had originally interpreted the most rapidly moving spot normally present in chromatograms from blood extracts as being due to the acetoacetate hydrazone, whose $R_F$ value closely resembles that of the more rapidly moving pyruvate component. However, we have now satisfied ourselves that in blood extracts this third spot is due to the more rapidly moving pyruvate isomer, since in animals poisoned with sodium arsenite, and therefore exhibiting raised blood pyruvate levels, the third spot is increased in amount, whereas in alloxan-poisoned animals, in which the blood aceto-acetate level is raised, the third spot is not increased.

Figure 4 shows two chromatograms of extracts from blood samples taken from a normal subject and a patient with peripheral neuritis 60 minutes after a dose of glucose. In each case an amount of the blood extract equivalent to 0.5 ml. of blood was applied to the paper. It will be seen that in the polyneuritic patient both the pyruvate

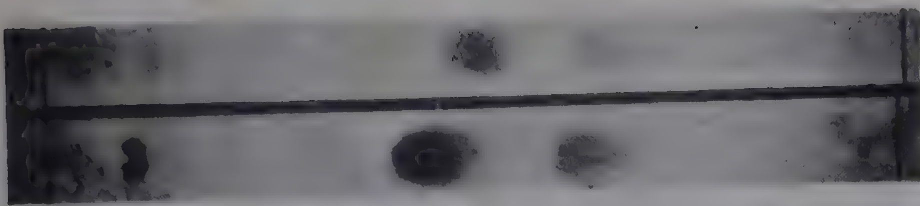

FIG. 4. — Chromatograms of α-keto acid hydrazones extracted from blood from a normal subject and from a patient with peripheral neuritis. Upper : normal (P. G. H.), 0.5 ml. blood, 6.3 μg. pyruvic acid. Lower : polyneuritis (D. S.), 0.5 ml. blood, 12.7 μg. pyruvic acid.

spots are increased in intensity, and also the α-keto-glutarate spot.

To return to subacute combined degeneration of the cord, using this chromatographic technique we have demonstrated that the high blood keto-acid levels, as determined by the method of Friedemann and Haugen, in these untreated cases of subacute combined degeneration of the cord are in fact due to pyruvate, α-ketoglutarate values being within the normal range, and no other keto-acids being detected on the chromatograms.

This finding is of interest in connection with the work of Dubnoff (21, 22) and others suggesting that vitamin $B_{12}$ may be intimately concerned with the reduction of disulphide compounds to the —SH form. Thus, the content of reduced glutathione of rat blood falls during a deficiency of vitamin $B_{12}$, and rises again on its re-inclusion in the diet. In pernicious anaemia in man the same has been shown, the blood —SH falling and rising again on treatment. It has recently been claimed that the reduction of co-enzyme A to the —SH form may be interfered with in vitamin $B_{12}$ deficiency, thereby inhibiting the normal metabolic path of both carbohydrates and fats. Consistent with this view, Ling and Chow (23) have described disturbances of carbohydrate and fat metabolism (hyperglycaemia and impaired glucose tolerance) in vitamin $B_{12}$-deficient rats.

It seems possible therefore that the degeneration of the peripheral nerves and long tracts in the cord in subacute combined degeneration, so similar in some ways to the changes occurring in thiamine deficiency, may be an example of a similar metabolic impairment, an inhibition of the normal rate of pyruvate oxidation by the neurones, but brought about by a different means, namely an interference with the $SS \leftrightarrows SH$ equilibria on which depends the adequate supply of co-enzyme A in the normal, functional state.

*Diabetic neuropathy*

Before leaving the subject of altered pyruvate metabolism in the neuropathies I want to say a few words about diabetic neuropathy. One of the several theories put forward to account for the development of peripheral neuritis in *diabetes mellitus* has postulated that this complication is due to an associated thiamine deficiency; another theory has suggested that it is the expression of the local reaction of the nerve cells to the underlying disorder of carbohydrate metabolism in diabetes. Pyruvate metabolism, both in uncomplicated diabetes and in diabetic neuropathy, has therefore received considerable attention. As is to be expected, the diabetic subject shows little or no rise in the blood pyruvate level following glucose administration, although a significant rise occurs when insulin is given as well (24). The interpretation of changes in the blood keto-acids in diabetes is rendered difficult, however, for two reasons. In the first place, the possibility of the presence of ketosis may lead to errors owing to varying amounts of aceto-acetate being estimated and recorded as pyruvate; and secondly, variations in the insulin-sensitivity of different patients may give rise to varying rates of pyruvate formation from glucose.

Smith and Taylor (25) have attempted to exclude the first of these difficulties, namely interference from acetoacetate, by the application of a more selective method involving the use of 1,2-diamino-4-nitrobenzene as a specific reagent for α-keto-acids, followed by paper chromatographic separation of the derivatives; they have concluded that in the well-controlled diabetic patient the levels of pyruvate and α-ketoglutarate in the blood are normal. In their report of these findings they did not, however, comment on the effects of injected insulin.

Markees and Meyer (26) and Martin (27) have each attempted to exclude the variable effect of insulin sensitivity on the rate of pyruvate formation from blood sugar by injecting sodium pyruvate intravenously, and comparing the rate of its removal from the blood stream in normal and in diabetic subjects. Unfortunately the results of these two investigations were not consistent, but it is safe to conclude that up to the present no clear-cut demonstration has yet been afforded of any serious impairment of pyruvate metabolism in diabetic neuritis. In support of this conclusion it must be remembered that Goodhart and Sinclair (28) found normal levels of blood co-carboxylase in four out of five patients with diabetic neuropathy, the fifth case, in whom a low level was found, being also an alcohol addict.

More recently, however, the question of the phosphorylation of thiamine in diabetes has been re-opened, and reports have appeared indicating that there may be defective formation of thiamine pyrophosphate in the diabetic liver (29, 30, 31).

In view of the possibility of increased adrenocortical activity being responsible for the production of certain types of *diabetes mellitus*, it is of interest that a number of workers have recently reported high blood pyruvate levels in patients treated with ACTH or cortisone. Thus, Kerppola (32) studied 32 patients, chiefly suffering from rheumatoid arthritis, and showed that after treatment for 5 to 7 days with the usual therapeutic doses of ACTH or cortisone the blood pyruvate rose to high levels. Gitelson (33) and Weissbecker and Schröter (34) have also reported similar changes. The connection between abnormal pyruvate metabolism and alterations in adrenocortical activity is further strengthened by the finding by Hills, Power and Wilder (35) of high blood pyruvate values in Cushing's syndrome, while Butterfield (36), in preliminary studies, has reported high values in insulin-resistant diabetics one hour after insulin was given in the glucose tolerance test.

So that even if it should ultimately prove that thiamine deficiency can be ruled out as a cause of diabetic neuropahty, it would certainly seem possible that abnormal

pyruvate metabolism, resulting from adrenocortical overactivity, may be associated with certain types of diabetes, and may therefore be related to the development of the lesions in the peripheral nerves.

### *Treatment with BAL*

In view of the possibility of disturbances of pyruvate utilisation arising from disorders of thiol metabolism in the nervous system a number of reports have appeared on the treatment of the various types of neuropathy with BAL.

Schneider in 1950 treated 22 patients with diabetic neuropathy with 100-200 mg. of BAL daily foı 14 days, and described significant subjective improvement in 12 cases. Among the 10 cases showing no improvement 4 had had symptoms of paraesthesiae and numbness for over 3 years, while 2 further cases had evidence of severe peripheral vascular insufficiency. Unfortunately the objective signs of improvement in this series were few.

BAL therapy has also been tried in other types of peripheral neuropathy. Furmanski (37) described the successful treatment of 4 cases of what he termed 'toxic' polyneuritis, but he did not report the blood pyruvate levels in these cases.

Joiner, McArdle and I also studied the effect of treatment with BAL in 6 cases of peripheral neuritis showing high blood pyruvate levels which had been unaffected by massive parenteral thiamine therapy. In 3 of these cases the blood pyruvate level became rapidly restored to normal, but the clinical responses of 2 of them were confused by other complicating factors. It is of interest, however, that two of these three cases gave a history of occupational exposure to hazards of heavy-metal intoxication.

### *Acute porphyria*

More recently a detailed case-report has appeared on BAL therapy in a case of acute porphyria showing severe motor paralysis, dysphagia and blurred vision (Peters, 38). The patient became unconscious, but consciousness was recovered 3 ½ hours after the first dose of BAL, and abnormal porphyrins disappeared from the urine in the course of a few days. When the BAL injections were stopped increased amounts of uroporphyrin reappeared in the urine and persisted until BAL therapy was resumed. Although little can be concluded from this single case, it is perhaps relevant to note that two cases of acute porphyria which we have studied at Guy's Hospital each showed blood pyruvate levels, after glucose administration, above the normal range.

On present evidence we have no reason for regarding porphyrins as neurotoxic; neither has porphobilinogen been shown to have any pharmacological action on the central nervous system (39). It would seem possible therefore that in acute porphyria there may be some widespread block in decarboxylation mechanisms which may cause both an interference with pyruvate metabolism, with consequent neurological changes, and a disturbance of the normal processes whereby uroporphyrin is successively decarboxylated to give coproporphyrin and protoporphyrin; as a result of this latter lesion the excretion of both porphobilinogen and of uro- and coproporphyrins might be increased. It would certainly seem that the neurological complications of acute porphyria would merit a further study along these lines.

### *Tri-*ortho*-cresyl phosphate paralysis*

I want now to turn to the first of the biochemical types of peripheral neuropathy that I mentioned earlier. That is to say, the large and important group of cases in whom no abnormality of pyruvate metabolism can be detected by the usual method of determining blood pyruvate levels after a loading dose of glucose. Since in many of these cases there is no evidence of any infective origin, it may be presumed that, if they are indeed metabolic in origin, the biochemical disorder must therefore be of a very different nature from those we have considered so far.

Concerning the nature of the disorder in these cases we have as yet little information, but perhaps a clue has been given, although a distant one, in recent work on the flaccid paralysis caused by intoxication with tri-*ortho*-cresyl phosphate (TOCP) and certain other organo-phosphorus compounds.

Tri-*ortho*-cresyl phosphate has for many years been recognised as a neurotoxic compound, having been shown to be responsible for the widespread outbreak of so-called ginger paralysis in the United States in 1930 (40, 41, 42). Since then other outbreaks of paralysis following ingestion of this compound have been described in Holland, France, Germany, South Africa and England.

On the assumption that the motor paralysis induced by this compound might be associated with a lesion at the motor endplates in the affected muscles, Bloch (43) began a study of the action of tri-*ortho*-cresylphosphate, or TOCP, on cholinesterase activity. He showed that horse serum cholinesterase was inhibited by low concentrations of this substance, and suggested that the flaccid paralysis observed in TOCP poisoning might be due to inhibition of the cholinesterase at the motor endplates, leading to a local accumulation of acetylcholine, which would in turn produce a neuro-muscular block.

Hottinger and Bloch (44) next showed that brain cholinesterase is inhibited by TOCP, and also that tributyrinase activity is diminished by it, although alkaline phosphatase, trypsin, arginase, histidase and pancreatic lipase are unaffected.

The nerve degeneration which is found both peripherally and centrally in TOCP poisoning and the delay, of about 14 days before the onset of symptons, are, however, difficult to explain on the basis of inhibition of cholinesterase at the motor endplates being the causative lesion.

At the time of Bloch's original work selective substrates for differentiating between true and pseudo-cholinesterase were not available. Later however Mendel and Rudney, in a brief report (45) showed that the administration of TOCP to rats causes a lowering of the serum pseudocholinesterase, but does not affect the true cholinesterase of the erythrocytes. The rat, however, is not sensitive to poisoning by TOCP, and in view of this it seemed desirable to investigate in greater detail the

action of TOCP on the different types of cholinesterase in a species known to develop nerve lesions following poisoning by this compound. When this was done, using the tissues of the hen which had earlier been shown to be highly sensitive to poisoning by TOCP it was found that while the pseudocholinesterase present in the central nervous system and in peripheral nerve was markedly inhibited by concentrations of the order of $10^{-6}$ M, the true cholinesterase at the motor endplates or in nervous tissue or erythrocytes was not significantly affected (46).

It was next shown that pseudo-cholinesterase is also inhibited *in vivo* by TOCP. After poisoning hens with a single dose of 1 ml. TOCP/kg. it was found that a sharp fall in the level of the serum cholinesterase is produced by the end of the first day, the activity thereafter gradually returning to the normal level over the next 14 days. In the spinal cord of poisoned hens the same change is found, a marked depression of pseudo-cholinesterase activity, with only a very slow return towards the normal level; the true cholinesterase of the spinal cord is only very slightly affected, if at all (47).

These findings, therefore, do not support Bloch's original view that paralysis is due to inhibition of the true cholinesterase at the motor endplates, or even at synapses in the central nervous system. They show, instead, an inhibition of pseudo-cholinesterase.

If, however, this inhibition of pseudo-cholinesterase is important in the production of the paralysis, then it might be expected that other selective inhibitors of this enzyme might also cause a similar paralysis. And it has now been shown that certain other organo-phosphorus anti-cholinesterases which are in use as insecticides are capable of producing paralysis and degenerative changes in the nervous system very similar to those caused by TOCP.

Petry (48) first described a case of paralysis occurring in a man who had been exposed to the insecticide parathion, diethyl-*p*-nitrophenyl thiophosphate. Two years later, two cases of paralysis occurred as a result of occupational exposure to another organo-phosphorus compound, 'mipafox' or bis-(mono*iso*propylamino)-fluorophosphine oxide (49). In these two cases the initial symptoms of acute cholinergic overactivity, which came on a few hours after exposure to the toxic compound, subsided with treatment, but 10-14 days later neurological symptons developed and progressed rapidly to give a severe flaccid paralysis of the extremities. Clinically, the patients were said to resemble very closely the picture in tri-*ortho*-cresyl phosphate poisoning.

The blood pyruvate level in these patients after glucose administration was normal, but both the plasma and erythyrocyte cholinesterases were profoundly reduced.

Barnes and Denz (50) next succeeded in producing paralysis in hens with this compound, and also with DFP, di*iso*propyl phosphorofluoridate, and showed that each of these compounds produces widespread degeneration and demyelination both of the sciatic nerves and of the long tracts in the spinal cord. More recently, Barnes (private communication) has also produced similar changes with diethyl phosphorofluoridate.

All of these compounds capable of producing these chronic lesions in the nervous system are selective inhibitors of pseudo-cholinesterase. But we cannot as yet relate their neurotoxic action in any direct way to inhibition of this enzyme, since there are other organo-phosphorus anti-cholinesterases, quite similar in chemical structure, which do not cause paralysis or degenerative lesions of the nerves even although they may be capable of producing an extensive degree of inhibition of pseudo-cholinesterase in the spinal cord after administration to animals (51, 52).

If therefore inhibition of this enzyme is playing any part in the production of these lesions it is clear that other factors must also be concerned. Histologically, the changes produced by these compounds in the hen resemble very closely the changes occurring in the nervous system of the thiamine-deficient pigeons. But no evidence has as yet been produced suggesting that there is any interference with the action of thiamine in this type of neuropathy. Indeed we have found the blood pyruvate level and the rate of oxidation of glucose and of pyruvate by brain to be normal in animals poisoned with TOCP; neither have we found DFP to have any action on the thiamine pyrophosphatase of brain or on oxidative phosphorylation by brain in concentrations even greater than are likely to occur in the nervous system of poisoned animals (53). Further Fenton (private communication) has been unable to influence either the time of onset or the severity of the paralysis by the administration of large doses of thiamine, vitamin $B_{12}$ or folic acid or by the giving of thyroxine.

Cavanagh and I have recently tried the effect of large doses of co-carboxylase, but we have found that 10 mg./day injected intramuscularly into the hen again had no effect on the time of onset or severity of the paralysis induced by a single dose of 1 ml. TOCP/kg. body weight.

We have therefore, as yet, no clear understanding of the biochemical lesion in the motor paralysis produced by these compounds. As I have already pointed out, the only biochemical feature which these 'demyelinating' anticholinesterases have in common is that they are all inhibitors of certain esterases, and in particular of pseudo-cholinesterase. Our further interpretation of the mechanism of this toxic effect is hampered both by our ignorance of the physiological function of pseudo-cholinesterase, and by the multiplicity of different esterases which are present in the nervous system.

With regard to the function of pseudo-cholinesterase in the central nervous system it is, at the present time, far from clear whether, like the true cholinesterase, it is concerned with acetylcholine metabolism and cell excitation or whether it participates in some quite different metabolic process. Rothenberg (54) and Grundfest and Nachmansohn (55) have suggested that the acetyl-choline-cholinesterase system may be concerned with the maintenance of the normal ion permeability of the axon of the squid nerve. On the other hand, work which we have recently carried out on chicken brain slices has indicated that with this tissue the leakage of potassium brought about by high concentrations of inhibitors of pseudo-cholinesterase is due to some other effect, possibly an action on the mechanisms of oxidative phosphorylation in the brain cells (56).

Evidence is, however, now appearing that pseudo-cholinesterase may, in certain sites at any rate, influence the processes of cell excitation in a similar manner to that exerted by the true cholinesterase at nerve synapses or at the motor endplates.

Burn and Walker (57) for example have studied the actions of selective inhibitors of true and pseudo-cholinesterase on the heart rate in the heart-lung preparations of the dog. Although both types of cholinesterase are present in the heart, pseudo-cholinesterase is present in much greater amount (58) and Burn and Walker (57) have demonstrated that inhibition of the pseudo-cholinesterase leads to slowing of the heart, whereas inhibition of the true cholinesterase does not.

Furthermore, we have shown that after section of the sympathetic nerves to the rabbit's ear, the pseudo-cholinesterase activity of the central artery of the ear is reduced to about half that of the normally innervated artery ; acetylcholine also disappears from the denervated artery, which, as is well known, shows a heightened reactivity to constrictor stimuli. These observations suggest the presence of a sympathetic cholinergic nerve mechanism in the ear artery which involves the activity of a pseudo-cholinesterase (59).

And thirdly, in the proceedings of the last meeting of the British Physiological Society, Desmedt and La Grutta (60), of the *Laboratoire de Pathologie générale* of the University of Brussels, described experiments in which they studied the action of selective inhibitors of the cholinesterases on the electrical activity of the cerebral cortex. Their findings indicated that the activation of brain potentials depend on the inhibition of pseudo-cholinesterase. As there is evidence that, in the central nervous system, this enzyme is associated with the neuroglia, they have suggested that acetylcholine in the brain, besides its possible role as a transmitter at certain synapses, might act as a 'local hormone' and, by influencing the activity of the astrocytes or oligodendrocytes, might so affect the chemical milieu of the neurones.

Whether this be so or not, however, we are left with the problem that it is only certain of these esterase inhibitors which exhibit these more chronic neurotoxic actions, and this brings us back to the second of the two gaps in our knowledge in this field, which I mentioned earlier ; namely, the multiplicity of different esterases which are almost certainly concerned in the turn-over of the lipid-rich myelin. One of the characteristics of the neuropathy induced by tri-*ortho*-cresyl phosphate and these other compounds is the delay of 12 to 14 days before the appearance of symptoms. One possible explanation of this delay might be that the neuronal dysfunction develops as a result of a primary action on the myelin sheath, leading to the slow, simultaneous development of changes in both the sheath and the axon.

From this point of view, and also because of the outstandingly important problem of multiple sclerosis and the other demyelinating diseases, it would seem that a detailed study of the metabolism of the various myelin lipids is overdue. At the present time we have relatively little information about the lipases and esterases, other than the cholinesterases, of the nervous system. It is true that enzymes capable of attacking lecithins, cephalins, sphingomyelins and cerebrosides *in vitro* have all been described in brain, and more recently Sloane-Stanley (61) has demonstrated the presence of a somewhat more active enzyme that hydrolyses diphosphoinositide. But we have as yet little insight into the role that these enzymes may play in the life of the nerve cells and glial cells of the central nervous system.

Our knowledge of the phosphatases of the nervous system has recently been considerably advanced by histochemical studies, but it should perhaps be remembered that most of this work has been done with such unphysiological esters as phenyl phosphate or α-naphthyl phosphate, and I have only been able to find one report by Baccari (62) on the action of phosphatases of the nervous system on phosphoryl choline, a component of both the lecithins and the sphingomyelins. Yet phosphoryl choline, phosphoryl ethanolamine, phosphoryl serine and inositol monophosphate, which can be regarded as naturally occurring 'lipid phosphate esters' are of unusual interest as phosphatase substrates, in that, unlike the glycerophosphates or the aromatic phosphate esters, we have found that while they are readily hydrolysed by the alkaline phosphatase, they are virtually not attacked by the acid phosphatase of brain.

But, despite these gaps in our understanding of the metabolism of myelin, our knowledge of general biochemistry, and of certain aspects of the biochemistry of the nervous system, is now in such a state that it can be profitably applied to the study of the pathology of many of the nervous diseases. A start has indeed been made in certain of these diseases and work in many different fields has pointed the way for further advances.

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# Etude de mélanges de protéines par analyse électrophorétique et immunoélectrophorétique en milieu gélifié (*)

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(*Reçu le* 30 *septembre* 1955)

Une des caractéristiques de la biochimie et des difficultés que l'on y rencontre est le fait que les produits naturels sont des mélanges, souvent très complexes, de substances peu stables ayant parfois des propriétés chimiques ou physiques très voisines. Ceci est particulièrement vrai dans le cas des liquides biologiques naturels ou d'extraits totaux de tissus riches en protéines. Un grand pas a été franchi depuis que Tiselius a mis au point l'électrophorèse. Sa méthode a permis d'obtenir des renseignements de grande importance justement parce qu'elle est applicable directement à des produits naturels. Depuis quelques années, diverses autres techniques d'électrophorèse, utilisant divers supports, tels que le papier, l'amidon, des dérivés de la cellulose, ont été décrites et certaines sont entrées dans la pratique courante. Parmi ces techniques, je réserve une place spéciale à celle décrite par Gordon, Keil, Sebesta, Knessel et Sorm, et qui est basée sur l'emploi d'un gel de gélose. En effet, le gel présente un certain nombre d'avantages par rapport au papier ou autres substances solides, car la proportion de liquide y est très grande, de l'ordre de 98 % ; de ce fait, il y a moins de chances de se heurter à des questions d'adsorption ou d'effets dus à l'interface liquide/solide. L'électrophorèse en milieu gélifié se rapproche donc beaucoup de l'électrophorèse en milieu liquide et possède, de plus, l'avantage de ralentir la diffusion libre des substances et de faciliter ainsi, éventuellement, leur séparation ultérieure.

Mais, quelle que soit la technique d'électrophorèse utilisée, on doit se demander quel est le pouvoir séparateur et la sensibilité de la technique employée ? En d'autres mots, la méthode permet-elle de séparer ou de distinguer deux substances présentes à une faible concentration ? Nous savons, par exemple, que dans l'électrophorèse en milieu liquide avec système optique, on obtient des diagrammes ayant des clochers asymétriques lorsqu'on est en présence de deux substances voisines, mais nous savons aussi que la sensibilité est seulement de l'ordre de 3 %.

Parmi les nouvelles techniques d'électrophorèse sur colonnes d'amidon ou de dérivés de la cellulose, il en existe qui permettent des dispersions assez considérables des constituants d'un mélange. Il semble que ces techniques peuvent rendre de très grands services. Mais, même dans ce cas, il est important de pouvoir se rendre compte de l'homogénéité des produits séparés. Il est admis qu'on ne peut affirmer l'homogénéité d'une protéine sur un seul critère. Cette homogénéité sera à l'inverse d'autant plus probable, qu'on aura utilisé, pour la démontrer plusieurs méthodes basées sur des caractéristiques différentes, par exemple, solubilité et activité biologique ou électrophorèse et teneur de la macromolécule en un constituant particulier.

Ces considérations nous ont amené à élaborer une méthode qui fait appel à deux propriétés distinctes : la mobilité électrophorétique et la spécificité immunochimique.

Il est certainement inutile d'insister sur la spécificité des réactions immunochimiques et sur leur extrême sensibilité. Grâce aux travaux de Heidelberger et de son école, l'aspect quantitatif des réactions de précipitation spécifique est connu et on est maintenant en mesure de dire que la sensibilité de ces réactions est de l'ordre du microgramme. De ce fait, l'utilisation des réactions immunochimiques permet, même lorsqu'on ne possède que peu de substances, d'y déceler de petites impuretés. On peut trouver, par exemple, en utilisant un échantillon de quelques milligrammes, une impureté qui ne constitue que 0.1 %.

Nous avons donc élaboré, avec mon collaborateur C. A. Williams, une méthode que nous appelons « immunoélectrophorétique ». Son principe est le suivant (figure 1) : un petit échantillon du mélange à étudier est soumis à une électrophorèse dans un gel. Lorsque la dispersion électrophorétique est jugée suffisante, on fait diffuser perpendiculairement à l'axe de migration électrophorétique un immunsérum contenant des anticorps précipitants, spécifiques des constituants antigéniques du mélange étudié. Lorsque ces antigènes et les anticorps se rencontrent en proportions convenables, il y a formation de

(*) Conférence faite le vendredi 5 août au cours d'une séance commune aux sections 2 (chimie et physico-chimie des protéines et polypeptides), 10 (microbiologie chimique) et 14 (pathologie chimique et immunochimie). Le programme de cette dernière section avait été établi en accord avec l'Association internationale de Chimie clinique.

complexes insolubles, et on voit apparaître dans le gel des bandes ou lignes de précipités spécifiques sous forme d'arcs, comme dans la méthode d'Ouchterlony.

Grâce à la spécificité de ces réactions, chaque antigène forme une ligne indépendante ; on peut donc dénombrer les constituants d'un mélange et les définir d'après leurs mobilités, c'est-à-dire d'après leurs positions sur l'axe de migration. Il est, en effet évident que l'endroit où l'arc de précipitation se rapproche le plus de la source des anticorps correspond à la place où la substance était à son maximum de concentration. On peut ainsi établir les mobilités relatives des constituants d'un mélange et si parmi ces constituants il existe, par exemple, deux substances dont les mobilités sont exactement connues, on peut, par extrapolation calculer les mobilités des autres constituants du mélange.

Citons enfin encore un avantage de la méthode : les quantités de substances mises en œuvre peuvent être petites, puisque les réactions immunologiques sont très sensibles et puisque l'on effectue les deux analyses sur le même échantillon.

Mais, comme toujours, il y a aussi des inconvénients : le plus important est inhérent au réactif même que l'on utilise, l'immunsérum. Il est obtenu chez un animal auquel on injecte la substance que l'on étudie. Or, les animaux répondent plus ou moins bien et il est difficile, sinon impossible d'obtenir des réponses uniformes. Même en hyperimmunisant les animaux on n'arrive pas toujours à obtenir une formation d'anticorps envers tous les constituants d'un mélange. Pour obvier à cet inconvénient, qui est d'ailleurs commun à toutes les méthodes immunologiques, nous hyperimmunisons plusieurs animaux et nous choisissons le sérum le plus riche en divers anticorps.

Un autre inconvénient est la possibilité de superposition ou de dédoublement des bandes de précipitation spécifique. On admet, en effet, que chaque ligne correspond à au moins un antigène. Or, on peut supposer cependant que deux lignes se confondent comme dans la double diffusion dans les gels (Kaminski). Mais pour arriver à un tel phénomène dans cette méthode, il faudrait non seulement que deux substances aient la même vitesse de migration, mais encore que les rapports des concentrations en antigène et en anticorps et les vitesses de diffusion soient les mêmes. C'est, au total, très peu probable.

Pour le contrôler, le mieux est d'employer plusieurs immunsérums différents ; les proportions d'anticorps étant variables d'un sérum à un autre, s'il y avait superposition de deux lignes avec un sérum, il y a beaucoup de chances qu'elle n'ait pas lieu avec un autre.

Le dédoublement des lignes de précipitation, c'est-à-dire la formation de deux ou plusieurs lignes, même lorsqu'on a affaire à un seul antigène homogène, a été surtout observé dans la double diffusion dans les gels par Burtin, Mme Kaminski et Salvinien. Nous l'observons également dans la méthode immuno-électrophorétique. Cependant, si dans les méthodes utilisant la diffusion dans des gels, sans séparation électrophorétique préalable, il peut être parfois difficile de faire la différence entre la présence de deux lignes de précipité indépendantes, dues à la présence de deux systèmes précipitants, et le dédoublement non spécifique, dans le cas de la méthode immuno-électrophorétique cette difficulté s'estompe : si l'on a affaire à deux constituants voisins, mais indépendants, on a deux arcs de précipités dont les maxima ne correspondent pas (c'est-à-dire que les antigènes n'ont pas des mobilités identiques) ou les deux arcs sont nettement séparés et plus ou moins parallèles. Par contre, nous admettons qu'il s'agit d'un seul antigène lorsqu'on observe d'abord un arc, qui s'épaissit ensuite et se divise en donnant plusieurs lignes ; dans ce cas, les maxima des arcs se trouvent au même endroit et, généralement, les extrémités des arcs sont confluents.

Pour expliquer le dédoublement ou la multiplication des bandes de précipité spécifique dans les gels plusieurs hypothèses peuvent être envisagées : précipitation périodique du type des anneaux de Liesegang, hétérogénéité des anticorps, présence ou formation de complexes antigène-anticorps solubles. Aucune de ces hypothèses n'ayant reçu encore de preuves complètes, nous n'insisterons pas sur cette question.

Au cours de l'élaboration de la méthode immuno-électrophorétique, nous avons été amenés à mettre au point et à préciser la technique d'électrophorèse simple en milieu gélifié. Cette technique présente un grand intérêt par sa simplicité et par un certain nombre d'avantages par rapport à l'électrophorèse sur papier. En effet, si après la fin de l'électrophorèse en milieu gélifié, comme l'ont décrit mes collaborateurs Uriel et Scheidegger, on fixe les produits par un réactif approprié, puis on sèche le gel avec du papier filtre, on obtient une sorte de feuille parfaitement transparente ; on peut alors utiliser toutes les techniques histo-chimiques de coloration afin de caractériser les divers constituants du mélange et, le fond étant transparent, effectuer des mesures photométriques. Ces feuilles peuvent ensuite être décollées du verre et se conservent très bien.

On peut aussi, pour caractériser l'emplacement dans le gel d'une substance qui existe dans le mélange initial, utiliser après l'électrophorèse à la place d'un immunsérum, d'autres réactifs, comme par exemple un substrat lorsqu'on étudie un enzyme.

On peut enfin découper après la fin de l'électrophorèse le gel en petites tranches et ensuite, soit en extraire les produits ainsi séparés, soit effectuer divers dosages.

Des essais d'extraction ont déjà été réalisés, nous y reviendrons, des essais de dosages sont en cours d'exécution ; il est trop tôt pour pouvoir en discuter les résultats.

La réalisation pratique de la méthode est assez simple. Jusqu'à présent deux substances gélifiantes seulement ont pu être utilisées : toutes les recherches préliminaires et toutes les applications réalisées jusqu'à maintenant ont été faites avec la gélose. Dans des recherches encore inédites, faites avec le professeur Nowinski à Galveston, nous avons étudié l'emploi de la pectine. Cette substance présente certains avantages sur la gélose. En effet, on arrive à faire des gels ne contenant que 0.5 % de pectine en faisant agir de la pectine-estérase en présence d'ions $Ca^{++}$. Ces gels contiennent donc 99.5 % de liquide ; ils sont parfaitement transparents et on n'a pas besoin de chauffer, comme c'est le cas pour la gélose. D'autre part, à la fin de l'électrophorèse, on peut découper le gel et hydrolyser la pectine par une pectinase ; on n'est pas ainsi obligé d'effectuer une élution ou une extraction du gel, ce qui peut rendre de grands services.

Parmi les autres substances formant de vrais gels transparents, celles que nous avons essayées n'ont pas donné de résultats satisfaisants. Dans ce qui suit, je ne parlerai que des gels de gélose.

Les gels de gélose, généralement de 1.5 %, dans le tampon choisi, sont formés sur des plaques de verre photographique ; des bandes de papier-filtre servent de connexion aux vases-électrodes. L'appareillage est fort simple, analogue à celui employé pour l'électrophorèse sur papier.

Pour diminuer la diffusion libre des constituants du mélange, nous tâchons de réduire le temps d'électrophorèse en opérant avec une chute de potentiel relativement élevée, de l'ordre de 4 à 5 V/cm. Et pour éviter une trop grande évaporation due au chauffage du gel par le passage du courant, nous opérons avec des tampons dilués, par exemple de force ionique 0.03 à 0.05. Dans le but de ne pas compliquer l'appareillage nous n'utilisons pas de système réfrigérant et travaillons à la température du laboratoire. Dans les conditions empiriquement établies que je viens de résumer, nous obtenons de bons résultats, bien qu'il y ait contraction du gel. Cette contraction est due en partie à l'évaporation, mais aussi à l'électroendosmose qui semble inévitable. Bussard a proposé de réduire l'évaporation en recouvrant la surface du gel par une mince pellicule de matière plastique, obtenue en étendant une solution concentrée de cette substance dans un solvant volatil. Mais si cette pellicule n'adhère pas parfaitement à la surface du gel on risque d'avoir des irrégularités dans l'électrophorèse par suite de la formation de couches liquides entre le gel et la pellicule.

L'électroendosmose provoque un mouvement du liquide dans le sens contraire au courant ; ce mouvement est important et nous oblige généralement de placer l'échantillon à examiner au centre de la plaque de gélose. L'expérience a montré qu'il est préférable de mélanger cet échantillon avec de la gélose fondue car la diffusion est ainsi plus régulière et le gel présente une parfaite continuité. Mais cette manière d'opérer possède l'inconvénient que la substance à examiner, lors du mélange avec la gélose fondue, subit un chauffage aux environs de 40-50° C. L'utilisation de pectine, qui se gélifie sans chauffage, sous l'action de l'enzyme, permettra d'éviter le risque de dénaturation lié au chauffage de la gélose.

L'enregistrement des résultats peut être obtenu, soit sans traitement préalable par la photographie directe sur papier photographique ou sur film, soit après séchage du gel et coloration des précipités spécifiques. Comme dans l'électrophorèse simple en gélose, on peut appliquer ici des méthodes histochimiques pour caractériser diverses substances.

Récemment, Scheidegger a montré que pour étudier de très petites quantités de substances on peut utiliser une micro-méthode immuno-électrophorétique. On prépare, par exemple, des gels sur des lames porte-objet du microscope et on peut obtenir des résultats rapides, car les distances entre les emplacements des antigènes et la source d'anticorps étant très petites, la précipitation se fait en peu de temps. Pour la lecture des résultats il faut se servir d'une loupe ou agrandir les photographies.

## EXEMPLES DE RÉSULTATS

La méthode immuno-électrophorétique a été utilisée dans un certain nombre d'études, surtout dans l'étude des sérums normaux et pathologiques, mais aussi des constituants de l'œuf, du cristallin de l'œil, du liquide céphalo-rachidien, etc...

### *Sérum humain normal*

La plupart de nos études sur le sérum humain normal ont été faites avec C. A. Williams. Nous avons utilisé surtout un sérum d'un cheval (n° 31) hyperimmunisé par du sérum humain normal et très riche en anticorps différents. Quelques autres immunsérums (de lapins, de poules et d'ânes) ont été également préparés. Nous pouvons, à l'aide de ce sérum de cheval, distinguer dans le sérum humain normal (figure 2, A) 16 à 18 constituants. Dans l'ordre de mobilité décroissante nous distinguons maintenant : un ou, presque sûrement, deux constituants migrant plus vite que la sérumalbumine ; nous les désignons par la lettre grecque $\rho$. Vient ensuite la sérumalbumine qui donne une bande de précipitation qui s'élargit, se dédouble souvent, ou disparaît, car il y a beaucoup de sérumalbumine et on est en excès d'antigène. Sous la ligne de la sérumalbumine on observe une petite bande dont le maximum est sensiblement identique à celui de la sérumalbumine ; nous désignons ce composé provisoirement par la lettre X et tout ce que nous savons de ce composé est qu'il n'est pas contenu dans la fraction V de Cohn, car lorsqu'on épuise l'immunsérum par cette fraction, qui est de la sérumalbumine, la ligne de précipitation du constituant X subsiste (nous y reviendrons). Viennent ensuite les globulines $\alpha_1$ ; on en distingue 2 ou 3. Dans la zone des globulines $\alpha_2$, on arrive à dénombrer jusqu'à 5 lignes et dans la zone des globulines $\beta_1$, 3 ou même 4. On distingue ensuite une bande allongée des $\beta_2$ qui s'étend vers des mobilités plus grandes et enfin une très longue ligne qui correspond aux globulines $\gamma$. La ligne des $\beta_2$ n'est nettement visible que lorsqu'on supprime la ligne des $\gamma$-globulines en absorbant l'immunsérum par une préparation de $\gamma$-globulines pures (figure 2, A). L'allongement de la ligne des $\gamma$-globulines est très caractéristique et nous sommes arrivés à prouver que les globulines $\gamma$ forment une famille de protéines ayant la même spécificité antigénique, mais toute une série de mobilités allant jusqu'à 3-3.5 (pH = 8.2, tampon véronal). Il y a donc dans le sérum des protéines qui, d'après leurs mobilités, devraient être classées comme des globulines $\beta_1$, voire même $\alpha_1$, et qui possèdent les propriétés immunochimiques des $\gamma$-globulines, c'est-à-dire que leurs configurations chimiques sont semblables ou identiques à celles des globulines $\gamma$ ayant des mobilités classiques.

Lorsqu'on veut étudier la partie centrale de notre diagramme, on peut prolonger le temps d'électrophorèse. On distingue alors mieux les $\alpha$ et $\beta$-globulines et la partie rapide des $\gamma$-globulines. Il est souvent difficile, sinon impossible, de faire apparaître toutes les lignes en même temps, car pour certains composés on est en excès d'antigène ou d'anticorps, donc dans des proportions telles qu'il y a formation de complexes solubles.

Je me permets d'insister sur l'importance du fait qu'il existe de nombreuses globulines $\alpha$ et $\beta$ ; cela prouve que

sous les clochers que l'on observe dans l'appareil de Tisélius il y a plusieurs protéines différentes et que les mobilités différentes des maxima de ces clochers que l'on trouve avec différents sérums sont dues aux proportions, variables d'un sérum à un autre, de ces différentes protéines.

Parmi les nombreuses globulines $\alpha$ et $\beta$, nous avons pu identifier jusqu'à présent : la sidérophiline, l'haptoglobine et des lipoprotéines $\alpha$.

Une identification d'un des constituants d'un mélange est facile lorsqu'on possède un échantillon du produit pur. Dans l'étude, effectuée avec P. Burtin, de la sidérophiline, c'est-à-dire de la $\beta$-globuline qui se combine avec le fer, nous nous sommes servis d'un échantillon de cette protéine aimablement offert par le professeur Schultze. En développant une électrophorèse, d'une part, avec l'immunsérum total et, d'autre part, avec ce même sérum épuisé par la sidérophiline, on peut affirmer que la ligne qui manque dans ce dernier cas est celle du produit étudié. Nous avons ainsi trouvé que dans notre diagramme général, c'est la ligne des globulines $\beta_1$ les plus lentes qui correspond à la sidérophiline (Sid. : figure 2, A).

Par une technique identique nous avons pu, en collaboration avec Mlle Boussier, M. Burtin et M. Jayle, préciser la position de l'haptoglobine. L'haptoglobine décrite par Jayle est caractérisée par sa réaction avec l'hémoglobine et est très riche en glucides. Nous avons pu prouver que l'haptoglobine existe comme composé indépendant dans le sérum et la situer sur notre diagramme général : c'est une des deux globulines $\alpha_2$, les plus lentes (Hapt. : figure 2, A).

Utilisant des colorants des lipides, nous avons constaté avec Uriel que dans l'électrophorèse en gélose, les lipoprotéines du sérum se retrouvent dans trois zones : dans la fraction rapide $\rho$, dans la zone de l'albumine et dans celle des globulines $\alpha_1$, mais nous n'en avons pas trouvé dans la zone des globulines $\beta$, comme on le constate en faisant des électrophorèses sur papier. Les mêmes colorants des lipides appliqués dans l'analyse immuno-électrophorétique ont coloré des lignes de précipités spécifiques dans les mêmes endroits, ce qui prouve bien qu'il s'agit de lipoprotéines et non de lipides libres.

Pour démontrer que dans l'électrophorèse en gélose, la dispersion des différents constituants du sérum est due à leurs différentes mobilités et que ces mobilités sont bien caractéristiques et constantes, nous avons effectué, avec Mlle Courcon, l'expérience suivante : du sérum humain a été soumis à une électrophorèse dans une plaque de gélose. Cette plaque a été alors découpée en 50 petits morceaux ; ces morceaux ont été ensuite transférés sur des plaques de gélose fraîches et soumises à une nouvelle électrophorèse dans les mêmes conditions, suivie d'un développement par l'immunsérum de cheval. Les résultats ont montré qu'on arrive ainsi très facilement à isoler tous les différents constituants d'un sérum d'après leurs mobilités respectives et ont confirmé l'existence de $\gamma$-globulines ayant des mobilités caractéristiques différentes, de nombreuses $\alpha$ et $\beta$-globulines, les composants X et $\rho$, etc. Le seul composé qui a été entraîné, probablement à cause de sa plus grande vitesse de diffusion libre, est la sérumalbumine ; on la retrouve, avec sa mobilité caractéristique, dans des fractions migrant plus lentement.

La grande sensibilité de la méthode permet son emploi dans l'étude de divers fractionnements de mélanges de protéines ; on arrive très facilement à préciser les constituants des fractions isolées par des méthodes de fractionnement variées ou les impuretés présentes dans diverses fractions.

C'est ainsi que nous avons, avec Williams, examiné diverses préparations de $\gamma$-globulines humaines ; certaines étaient homogènes, d'autres contenaient diverses impuretés ($\beta$-globulines, albumine) ; diverses autres fractions ou sous-fractions ont généralement montré qu'elles sont des mélanges ; en utilisant l'immunsérum épuisé par des fractions connues, nous avons prouvé que la fraction IV-7, riche en $\beta_1$-globulines (surtout sidérophiline), contient de la sérumalbumine et des globulines $\gamma$.

### *Sérum de nouveau-né*

En collaboration avec Martin, Scheidegger et Williams, nous avons constaté que le sérum du nouveau-né ne contient pas de globulines $\beta_2$.

### *Sérums pathologiques*

Certains sérums pathologiques donnent des images très caractéristiques. Des recherches, poursuivies avec Burtin, Fauvert et Hartmann, nous avons reproduit trois diagrammes : augmentation très prononcée des $\gamma$-globulines lentes, dans un cas de myélome (figure 2, C) et des $\gamma$-globulines rapides, dans un autre cas de myélome (figure 2, D) ; mise en évidence de protéines anormales (figure 2, E).

### *Mobilités de divers anticorps*

La méthode immuno-électrophorétique permet aussi de se rendre compte des mobilités de divers anticorps. Dans ce cas, nous faisons l'électrophorèse de l'immunsérum que nous étudions et nous faisons ensuite réagir d'une part les antigènes et d'autre part un autre immunsérum spécifique du sérum étudié et qui permet de différencier les divers constituants. Dans un travail fait avec Williams, nous avons montré que dans un immunsérum de cheval, il peut exister des anticorps plus rapides que d'autres ou bien des anticorps ayant la même spécificité, mais deux mobilités différentes.

Dans une étude faite avec Faure, Fine, St-Paul et Eyquem, nous avons pu montrer que les hémagglutinines du sérum humain, séparées par électrophorèse en gélose, puis éluées de la gélose, ont des mobilités qui les feraient ranger parmi les globulines $\beta$. Or, ces hémagglutinines réagissent immunochimiquement comme des globulines $\gamma$. Nous concluons donc que ces agglutinines se trouvent, sur notre diagramme immuno-électrophorétique, parmi les globulines $\gamma$ les plus rapides, ayant des mobilités semblables au groupe des globulines $\beta$ (tableau I).

### *Produits de clivage de la sérumalbumine*

Dans une étude récente, Lapresle a pu montrer que la protéase, présente dans les cellules réticulo-endothéliales (rate, moelle osseuse), clive la sérumalbumine en des produits qui précipitent encore avec l'immunsérum de lapin anti-sérumalbumine. La méthode immuno-électrophorétique a permis de prouver qu'il y a trois produits du clivage qui possèdent des vitesses de migration

Tableau I

*Etude de la mobilité des hémagglutinines humaines par électrophorèse des sérums en gélose, suivie d'une élution des constituants (contrôle électrophorétique)*

| Anticorps | Sérum | Groupe sanguin | Elution | | | | | G. R. | Activité immunologique des éluats | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Quantité (µl.) | Durée (h.) | Température (°C.) | NaCl 0.85 % (volumes) | Centrifugation 30 min. (R. P. M.) | | -6 | -5 | -4 | -3 | -2 | -1 | 0 | +1 | +2 | +3 | +4 | +5 |
| Iso-hémagglutinines naturelles | Anti-A | B | 20 | 24 | 18–25 | 3 | 3 000 | A | — | — | — | ++ | ++ | + | ++++ | — | — | — | — | — |
| | Anti-A | B | 27 | 84 | 4 | 3 | — | A | — | — | — | +++ | + | + | ++ | — | — | — | — | — |
| | Anti-A | B | 27 | 72 | 4 | 2 | 10 000 | A | — | — | — | ++ | ++++ | ++++ | ++ | — | — | — | — | — |
| | Anti-A | B | 27 | 72 | 4 | 2 | 10 000 | A | — | — | — | ++++ | ++++ | +++ | +++ | — | — | — | — | — |
| | Anti-A | B | 20 | 16 | 18–25 | 2 | 10 000 | A | — | — | — | +++ | ++++ | +++ | +++ | — | — | — | — | — |
| | Absorbé par G.R. A. | B | 20 | 16 | 18–25 | 2 | 10 000 | A | — | — | — | — | — | — | — | — | — | — | — | — |
| | Elution | B | 20 | 16 | 18–25 | 2 | 10 000 | A | — | — | — | — | ++++ | +++ | — | — | — | — | — | — |
| Iso-immunagglutinines | Anti-A n° 8 | O | 33 | 16 | 18–25 | 2 | 10 000 | A | — | + | +++ | ++ | ++++ | +++ | + | — | — | — | — | — |
| | Anti-B n° 3 | A | 33 | 46 | 4 | 2 | 10 000 | B | — | — | — | +++ | ++++ | ++ | ± | — | — | — | — | — |
| | Anti-B n° 4 | A | 33 | 16 | 18–25 | 2 | 10 000 | B | — | — | — | ++++ | ++++ | ++ | ± | — | — | — | — | — |
| | Anti-B n° 7 | O | 20 | 16 | 18–25 | 3 | 10 000 | B | — | — | — | +++ | ++++ | +++ | ± | — | — | — | — | — |
| | Anti-B n° 8 | O | 33 | 16 | 18–25 | 2 | 10 000 | B | — | — | ++ | ++++ | ++ | ++ | ++ | — | — | — | — | — |
| | Anti-Rh (C+D) n° 1 | O | 20 | 20 | 18–25 | 3 | 10 000 | $O^+$ | — | — | — | — | — | ++ | + | ++ | — | — | — | — |
| | Anti-Rh (C+D) n° 1 | O | 20 | 20 | 18–25 | 3 | 10 000 | $O^+$ (papaïnisés) | — | ++ | + | + | ++ | ++ | ++++ | + | — | — | — | — |
| | Anti-Rh (C+D) n° 2 | A | 27 | 16 | 4 | 3 | — | $O^+$ (papaïnisés) | + | ++ | + | + | + | + | ++++ | +++ | — | — | — | — |
| | Cryoagglutinines | A | 27 | 84 | 4 | 3 | 10 000 | $O^+$ | — | — | — | + | ++ | ± | ++ | — | — | — | — | — |
| | Cryoagglutinines | A | 33 | 40 | 4 | 2 | 10 000 | A (papaïnisés) | — | — | — | ++ | +++ | +++ | ++ | — | — | — | — | — |
| | Mononucléose | — | 20 | 16 | 18–25 | 2 | 10 000 | mouton | — | — | — | ++ | +++ | +++ | ++++ | — | — | — | — | — |
| Immunoélectrophorèse { S-31 épuisé par γ / S-31 entier | | | | | | | | |  | | | | | | | | | | | |
| Electrophorèse simple | | | | | | | | | | | | | | | | | | | | |



Les chiffres indiquant les éluats correspondent aux distances (en cm.) à partir du réservoir du départ. Les colonnes sont disposées en regard des diagrammes électrophorétiques.

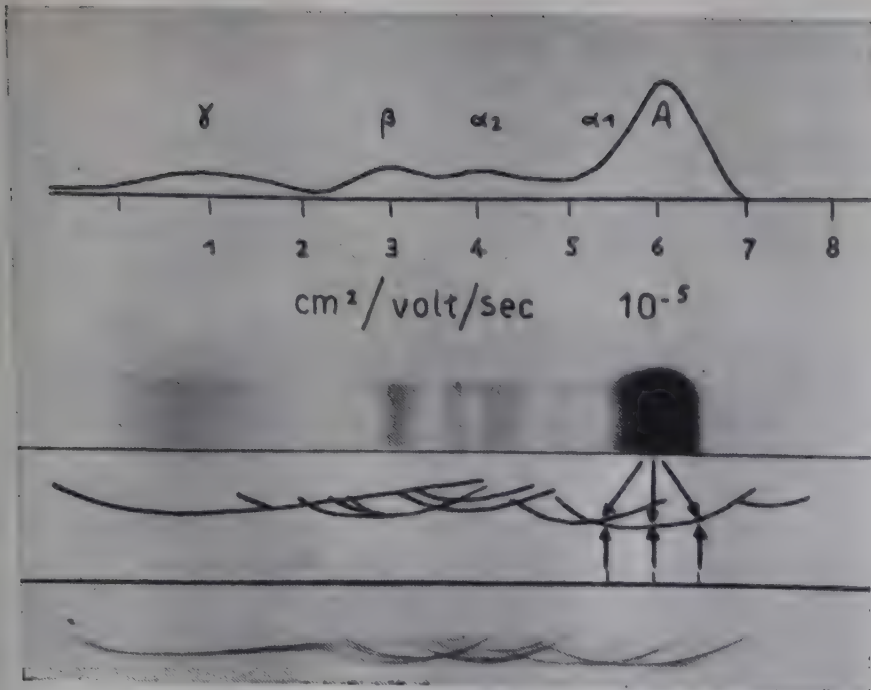


FIG. 1. — Sérum humain normal. De haut en bas : diagramme électrophorétique (en milieu liquide) ; électrophorèse en milieu gélifié, coloration des protéines par l'Amido-Schwartz ; schéma de la formation des arcs de précipitation spécifique dans l'analyse immuno-électrophorétique ; photographie des arcs formés dans une expérience, après leur coloration par l'azo-carmin.

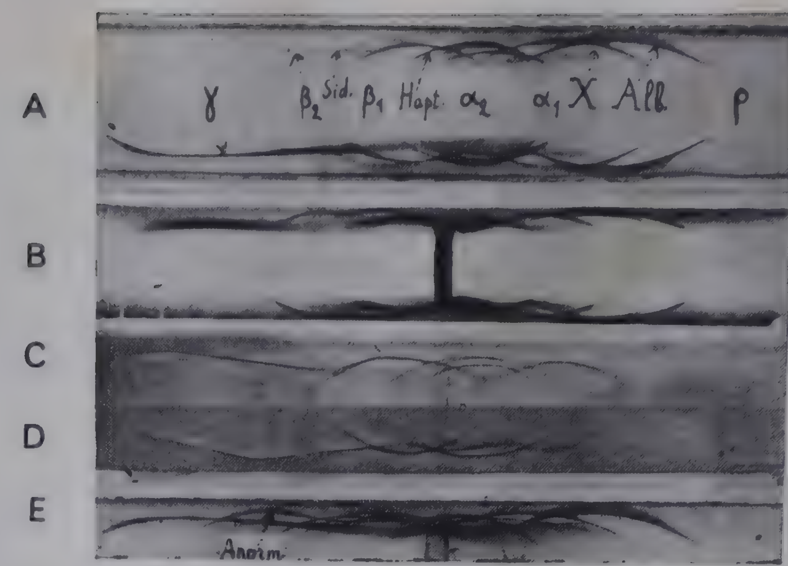


FIG. 2. — Analyses immuno-électrophorétiques. A : sérum humain normal, développé en bas par un immunsérum de cheval homologue, et, en haut, par le même immunsérum épuisé par des globulines γ. B : liquide céphalo-rachidien humain normal, développé en haut par le même immunsérum total et en bas par ce sérum épuisé par des γ-globulines. C, D et E : sérums humains pathologiques, développés par le même immunsérum de cheval. Conditions d'électrophorèse identiques.

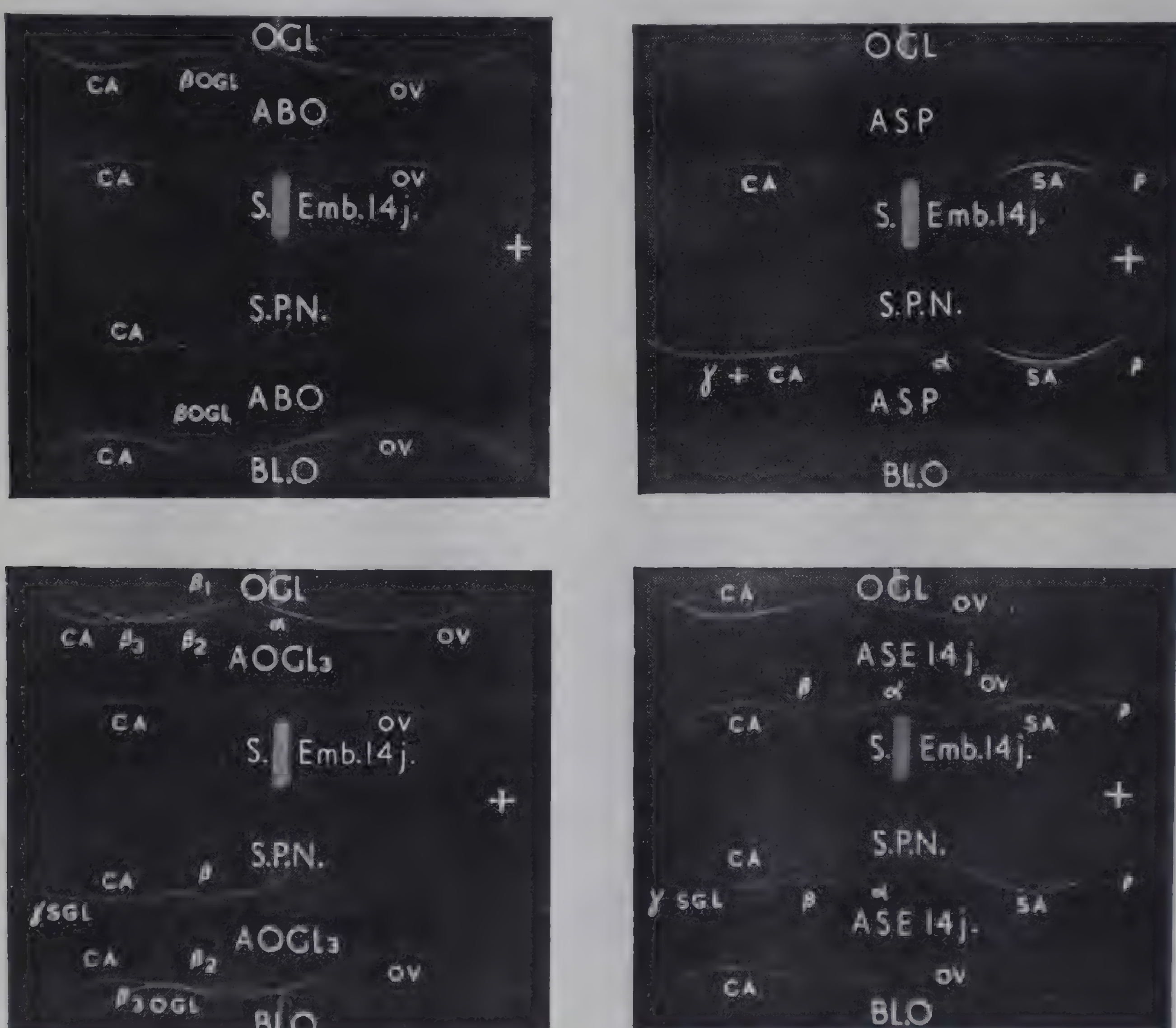


FIG. 3. — Analyses immuno-électrophorétiques de : Bl. O = blanc d'œuf ; OGl = ovoglobulines ; S.P.N. = sérum de poule normale ; S. Emb. 14 j. = sérum d'embryons de 14 jours d'incubation. Développements par des sérums de lapins : $AOGl_3$-anti-Ovoglobulines ; ASE-anti-sérum d'embryon ; ABO-anti-blanc d'œuf ; ASP-anti-sérum de poule.

différentes, puisqu'il y a trois lignes indépendantes. La molécule de sérumalbumine possède donc trois motifs antigéniques distincts et le lapin en réponse à l'injection d'un antigène homogène a formé trois anticorps ayant des spécificités différentes.

### *Blanc d'œuf, sérum de poule ou d'embryons*

Depuis quelques années, ma collaboratrice, Mme Kaminski poursuit, en partie avec Mlle Durieux, des études immunochimiques sur les constituants de l'œuf : blanc d'œuf, liquide amniotique et allantoïdien, sang de l'embryon et sérums de poussins, de poules et de coqs.

La figure 3 reproduit les résultats obtenus par des immuno-électrophorèses de 4 milieux différents : blanc d'œuf, sérum de poule normale, sérum d'embryons de 14 jours d'incubation et ovoglobulines, développées à l'aide des antisérums correspondants (sur chaque plaque les quatre mélanges d'antigènes réagissent avec un des quatre antisérums). On peut déduire de ces réactions qu'il existe des constituants communs à certains de ces milieux et des constituants spécifiques à chacun d'eux. Ainsi la conalbumine est-elle présente dans les quatre milieux ; l'ovalbumine est commune au blanc d'œuf, à la préparation d'ovoglobulines et au sérum d'embryons. La sérumalbumine existe dans le sérum de poule et celui d'embryons. D'autre part, la $\beta_3$-ovoglobuline ne se voit que dans le blanc d'œuf et dans la préparation d'ovoglobulines qui en provient. La $\gamma$-sérumglobuline est un constituant du seul sérum de poule. Un constituant plus rapide que la sérumalbumine se voit dans le sérum de poule et le sérum d'embryons. Il n'a pas la même mobilité dans les deux milieux, mais d'autres essais ont montré que la spécificité immunologique est unique.

### *Cristallin de bœuf*

Dans une étude sur les constituants du cristallin de bœuf (figure 4), Wieme et Mme Kaminski ont pu dénombrer au moins huit constituants à l'aide de la méthode immuno-électrophorétique. La photo n'étant malheureusement pas claire, nous ne reproduisons que le schema (figure 4) qui précise les positions relatives de ces divers constituants, dont certains n'ont pas pu être décelés par l'électrophorèse ordinaire.

### *Liquide céphalo-rachidien*

Et, comme dernier exemple, je me permets de mentionner un travail tout récent fait avec Mme Gavrilesco, Mlles Courcon et Hillion et MM. Uriel et Lewin ; nous avons étudié le liquide céphalo-rachidien humain. Lorsqu'on compare les diagrammes que l'on obtient avec le L.C.R. (figure 2, B) et le sérum (figure 2, A), on voit qu'il y a des différences très nettes. Je ne mentionnerai que trois faits : présence d'un composé rapide $\rho$ abondant ; pauvreté en globulines $\gamma$, qui sont d'ailleurs uniquement lentes, abondance et aspect particulier de la ligne des globulines $\beta_2$ : elle possède deux maxima de concentration, dont l'un est situé dans la zone des globulines $\beta_1$.

J'espère que cette série d'exemples d'applications de l'analyse immuno-électrophorétique m'a permis de montrer les diverses possibilités qu'elle offre. Je ne voudrais pas minimiser les quelques difficultés ou inconvénients que j'ai mentionnés, mais je crois qu'elle présente certains avantages et peut rendre d'appréciables services dans un grand nombre d'études sur les protéines.

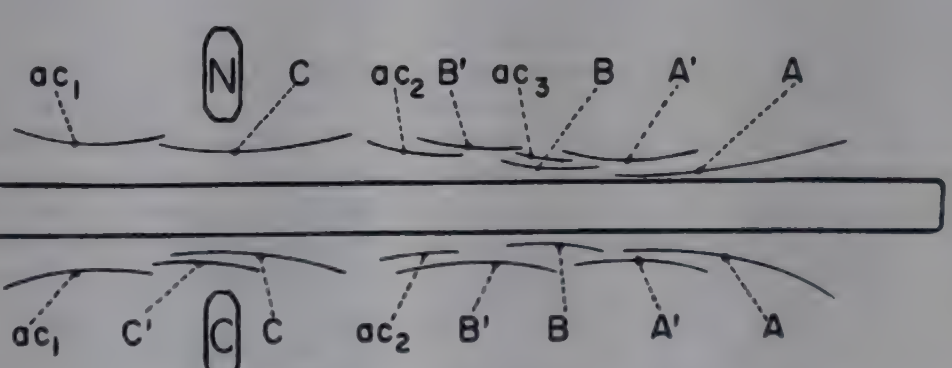


Fig. 4. — Analyse des constituants du cristallin de bœuf : reproduction schématique des arcs de précipitation spécifique.

# Microelements in nutrition, an approach to comparative biochemistry in mammals, including man (*)

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(*Received* 5 *August* 1955)

Presenting a paper on microelements in nutrition might be disappointing for the audience and discouraging for the reader, to a certain extent. For the time being thousands of data originating from older and recent experiments, mostly disconnected and carried out with greatly different views, are available. Collecting data is important, of course, but it is not the ultimate target of scientific research. We have to find out what is the background of the facts revealed. The different techniques used in these studies make a comparison of the results difficult, if not impossible. Due to inadequacy of methods used, many of the older data are conflicting and of no use to the apprehension and interpretation of the observations made. In the recent fifteen years the methods for the determination of microelements have been considerably improved, but despite the great advances made in this respect, it is, in very many cases, still not possible to interpret the mode of action of microelements in the human and animal organism, and to give a clear outline of their biochemical functions in normal and abnormal nutrition.

Pending a more thorough treatment of the various aspects of supply, requirements and functions of microelements in human and animal nutrition, which is in progress (1), it is not my intention to discuss the problem in general at present. For this would necessarily lead to a dry enumeration of disconnected and hence confusing facts. In the short time allotted to me I would rather discuss a few items from the standpoint of comparative biochemistry, in addition to what is recognized by scientific workers as the biochemical basis of the action of microelements *in vitro* and *in vivo*.

The first question that arises concerns the criteria of such a treatment. Since many observations are not at all clear and even confusing, I would direct attention to the differences observed regarding supply and requirements of microelements under various circumstances in life, such as growth, pregnancy, lactation, old age and disease. Furthermore attention has to be focused on the pathways of microelements metabolism in various species. This includes absorption, excretion and accumulation, the interaction of one microelement with another, the interrelationship of micro- and macroelements, and other food constituents, and finally the function of microelements in anabolic and catabolic processes under various circumstances. This is quite a program and it will be clear that a purposeful selection has to be made.

An attempt to give an overall picture of this kind is very often frustrated by lack of information. The main interest of many nutritionists concerns in the first instance microelements nutrition in human subjects, large domestic animals and poultry. However, experimental work has mainly been carried out on small laboratory animals and it is not justified to draw an analogy between different species without comment. On the other hand, certain disorders in which microelements are involved, are shown to be major problems in certain species, such as ruminants, whereas they hardly occur in man, and, if so, the features are greatly dissimilar, suggesting a different site of action of certain microelements in ruminants, as compared with other mammals.

It is my opinion that a biochemist active in the field of microelements research should keep close touch with experts in human and animal nutrition, for two reasons. A thorough knowledge of the specific phenomena related to biochemical functions of microelements *in vitro* and in general metabolism, may be greatly instructive so far as the interpretation of features in nutritional disorders is concerned. In that way the knowledge resulting from biochemical research may facilitate the nutritionist to making a sure diagnosis in disorders in which microelements are involved.

On the other hand, it happens sometimes that symptoms observed in human and animal nutrition, which are to be considered as a reflection of a number of partial effects of bio-catalyzers interfering with the normal course of intermediary metabolism, may point to disorders in which microelements might play a part and in this way be a hint for further biochemical research.

(*) Lecture given on Friday 5 August 1955 at a session of section 13 (nutrition). The programme of this section had been decided in collaboration with the International Association of Clinical Chemists.

*The biochemical basis of the action of microelements in nutrition*

All forms of life depend for their vital processes on the presence within them of a number of inorganic elements. These elements are derived from soil, water and air. In the first instance they are absorbed from these media by plants. All animals, including man, ultimately depend on plants and water for their mineral requirements. The inorganic elements have been grouped arbitrarily, mainly on the basis of the amounts required, in macro- and microelements. However, so far as the biochemical action of mineral elements in metabolic processes is concerned, it is sometimes difficult, if not impossible, to discriminate between macro- and microelements (1). Hitherto the following microelements are found to be essential for animals, including man, in varying quantities : iron, manganese, copper, zinc, iodine, fluorine, cobalt and molybdenum.

Evidence is available that microelements absorbed by mammals from the food may act on metabolic processes in three ways (1).

Firstly, they may be taken up in the molecules of certain enzymes, vitamins and hormones, these three groups of chemical compounds acting as bio-catalyzers in the processes of cell metabolism.

Secondly, they may act as activators of enzymes and in this way exert an influence on metabolic processes in the animal body.

Finally, there seems to be a third mode of action of certain ions, such as copper, as direct catalyzers in the transfer of oxygen, *e. g.* in the oxidation of easily oxidable substances like ascorbic acid.

Irrespective of these dynamic properties, certain microelements may have structural functions. This holds true, for instance, for fluorine in apatite crystals of bone tissue.

That the enzyme activity which is of predominant importance in metabolism is strongly enhanced by the presence of certain metal ions, is a well established fact (1).

This holds good for many, though not for all, peptidases present in animal (and plant) tissues which are shown to be activated *in vitro* by manganese, cobalt, iron, zinc and magnesium. In the enzyme arginase, that exerts a specific and important action in the last stage of protein breakdown, manganese is claimed to be the essential inorganic component in the enzyme complex, but the manganese-free compound is also reactivated by cobalt, nickel, vanadium and calcium.

Passing on from these *in vitro* experiments to experiments *in vivo*, I would particularly call attention to the action of cobalt, in the form of vitamin $B_{12}$, on amino acid and protein metabolism in higher animals. In his recent review of the present knowledge of the metabolic role of vitamin $B_{12}$, Porter mentions that a deficiency of $B_{12}$ causes accumulation of non-protein nitrogen in the blood (2). Such experiments suggest that the vitamin may aid protein synthesis in the tissues. This is in agreement with Henry and Kon's observation that the biological value of casein, and hence the assimilation of nitrogen, were significantly lower in rats deprived of vitamin $B_{12}$ than in rats receiving it (3).

In this connection experiments with rats initiated by Du Vigneaud and co-workers in 1939 and also carried out by several other groups of workers, have to be mentioned (4, 2). Evidence has been obtained, much of it from studies with $^{14}C$ and deuterium that the rat and the chick are able to synthesize the 'labile methyl groups' of methionine and choline from the tissue water and some one-, two- and three carbon compounds, in the presence of vitamin $B_{12}$ and folinic acid.

In the miscellaneous group of non-oxidative and oxidative enzymes, active in carbohydrate and fat metabolism, activation by metal ions such as zinc, manganese, vanadium, calcium and magnesium, is frequently observed, whereas in enzymes which are responsible for the transfer of oxygen, iron and copper come to the fore.

It is evident from experiments *in vitro* that copper plays an important part in the oxidation of *para*-cresol, pyrocatechol, hydroquinone and *para*-phenylenediamine by means of enzymes like 'tyrosinase', polyphenoloxidases and protein compounds such as coeruloplasmin, whereas diamino-oxydase (histaminase) which brings about the oxidative destruction of highly poisonous substances, such as histamine, cadaverine and putrescine, is activated by cobalt (5).

Finally it should be mentioned that the enzyme cholinesterase which exerts a specific action in the transfer of nervous stimuli is shown to be activated by manganese, baryum, magnesium and calcium.

So far as the practical side of the activation properties of microelements is concerned, two important facts should be mentioned.

— In many cases an optimal concentration of microelements is shown to exist with regard to the activation of enzyme systems. Both less and more of the elements in question may lower the speed of the enzyme action. This holds true for one enzyme separately, as well as for the intricate system that is termed 'the animal organism', in which a great number of enzymatic processes occur at the same time. Too low a concentration of microelements in the animal tissues may be attended with features of deficiency in the animal whereas excesses of microelements involve features of intoxication. Apparently this is a practical rather than a scientific discrimination, for in both cases, deficiency and excess, the clinical effects of intoxication observed in the animal, which are due to the accumulation of poisonous metabolites in the tissues originating from disturbances in the enzymatic processes, are prevalent.

— The fact that in many cases microelements act through their implication of enzyme action indicates that the pH of the body tissues will interfere with the function of microelements in the animal organism. Metabolic disorders collectively called acidosis and alcalosis will, in principle, exert an influence on the activity of microelements as bio-catalyzers, and *vice-versa*. In this connection Møllgaard and Thorbek's assumption that the acidosis after feeding A.I.V. silage in cattle is attended with a shift in the oxidation processes in favour of excessive heat production should be tested again (6).

A critical examination of the part played by microelements, and to a lesser degree by macroelements, in the animal body gives rise to the working hypothesis

that four kinds of processes of fundamental importance for normal life are subject to the action of these elements, to wit : (*i*) the synthesis and breakdown of tissue elements ; (*ii*) the energy producing processes, mainly oxido-reductions ; (*iii*) the detoxication of endogenous poisons ; (*iv*) the regulation of nervous stimuli.

It is the working-out of this hypothesis that may enable us to throw light upon so many questions in the field of nutrition where our insight fails at present.

Now I would proceed to a few questions of fundamental importance that are hitherto unsolved.

### *Balance trials as a tool to determine the requirements for microelements*

It stands to reason that the requirements of animals for microelements are strongly enhanced during pregnancy and growth. To some extent the same may hold true for lactation, namely so far as the milk secreted contains considerable amounts of microelements. The data available at the present time originate mainly from observations and estimations made in practice. Balance trials such as have been effected with iron and copper mainly in human subjects, are to be carried out in various species, under various circumstances in life. They would procure us the exact information we need. Up to the present, however, balance trials are very scarce so that no general outline can be given.

It is my opinion that this is the main reason why our insight into the requirements for microelements, particularly regarding large animals including man, is still very poor at the present time. This is an important terrain of future research.

### *Supply of microelements in vegetable and animal foodstuffs*

Although all animals, including man, ultimately depend on plants and water for their mineral requirements, food of vegetable origin is not always optimal to secure a normal supply of microelements. Concentrations may be both too high and too low to satisfy the requirements of the animals under various circumstances in life.

Selenium which is hitherto considered to be non-essential, may be taken up by some plants in very large amounts without damage to themselves, different species varying widely in this respect (7). Thus the botanically related *Astragalus missouriensis* and *Astragalus bisulcatus* grown on the same soil gave values of 3 and 1250 p.p.m. respectively. Such plants as the latter are selenium accumulators and selenium converters in the sense that they readily absorb the element from seleniferous soils and return it in due course to the soil in more soluble form for absorption by other plants. The question that forces itself on the biochemist is what determines the difference between the various plants, with regard to their absorption ability of selenium. This is a fundamental rather than a practical question, although answering this question in a satisfactory way might be of some practical use to solving major problems in the seleniferous areas where serious disorders in all stock including poultry occur.

Vegetable foodstuffs may also be deficient, for instance in cobalt in the form of vitamin $B_{12}$, so far as animals with a simple gastro-intestinal tract are concerned that cannot readily synthesize the vitamin themselves. In this case the passage of the element through animals, which are able to synthesize vitamin $B_{12}$ more readily, or to accumulate the vitamin in certain tissues that may be used as foodstuffs, is beneficial. In agreement with what might be expected, the meat and organs of the ruminant, namely the cow, were found to be higher in vitamin $B_{12}$ potency than those of pork (8).

That animal organs with high vitamin $B_{12}$ content, *e.g.* liver is now entirely uneconomic for large-scale production of the cobalt-containing vitamin $B_{12}$ is well kown in animal feeding. Commercial production is by fermentation, and the vitamin comes either from spent liquors in the manufacture of streptomycine and aureomycine, or it is made by a variety of micro-organisms in special fermentation. Here new avenues of research are opened.

Although milk is not an important source of vitamin $B_{12}$, a comparison of the $B_{12}$ content of milk from various species seems to be interesting from the viewpoint of comparative biochemistry. Comparing milk from the normal cow, goat, sheep, rat, dog, sow, horse and human subjects reveals that the vitamin $B_{12}$ content of the milk of non-ruminants is much more variable than that of the ruminants (9) (Table I).

TABLE I.

*Vitamin $B_{12}$ content of milk (µg./liter) from various species*

| | Mean | Range |
|---|---|---|
| Cow . . . | 6.6 | 3.2 — 12.4 (1 to 4) |
| Sheep . . | 1.4 | 1.0 — 2.0 (1 to 2) |
| Goat . . | 0.12 | 0.07 — 0.18 (1 to 2.5) |
| Human . | 0.41 | 0.10 — 1.5 (1 to 15) |
| Sow . . . | 1.05 | 0.03 — 2.7 (1 to 90) |
| Rat . . . | — | 11.0 — 95 (1 to 9) |
| Dog . . . | — | 0.70 — 13 (1 to 19) |
| Horse . . | 0.02 (one sample) | — |

After Collins, Harper, Schreiben and Elvehjem (9).

Whereas in ruminants the range varies up to a maximum of 1 to 4, it runs as high as 1 to 90 in the non-ruminants. Apparently the stabilization of the concentration of vitamin $B_{12}$ in the milk of normal non-ruminants is very poor in comparison with normal ruminants. These considerable variations are in part due to the dietary intake of the vitamin in the non-ruminants, whereas the ruminants are independent of the direct intake of vitamin $B_{12}$.

However, when comparing concentrations of the vitamin in the milk from the species mentioned, we are struck by two facts. First of all the vitamin $B_{12}$ concentration in the milk of the rat is four to eight times as high as that in the milk of the cow, the ruminant that shows the highest $B_{12}$ content in the milk. This difference cannot be explained from a high intake alone. Secondly from the three species of ruminants under investigation, cows, sheep and goats, goat's milk has a vitamin $B_{12}$ concentration that is only one fifth of the

concentration in cow's milk. Since the range of variation is small in both species, suggesting microbial balance in the rumen, the cause of this difference remains obscure.

It is my opinion that an important field of biochemical research lays fallow with respect to the factors responsible for the concentrations of vitamin $B_{12}$ in the milk, and their variations in various species.

### *Microelements in the biochemical regulation of body functions*

The normal state in the human and animal body is characterized by a high degree of constancy in the chemical composition of blood and other tissues. In functional disturbances this constancy may no longer hold good, with resulting variation in the chemical composition of blood and other tissues. In studying these problems the first question the biochemist asks himself is, how is the balance of functions, leading to the stationary condition, the so-called 'biochemical balance' in the organism, brought about ? The next is, which factors contribute to the disturbance of this balance with the occurrence of biochemical and clinical symptoms of disease ?

When we consider the different kinds of symptoms in the metabolic disorders which have been observed, we may point to the correlation of the numerous changes which evidently take place in the animal body, changes about whose complicated mechanism we are in many cases still in the dark. In this regulating system the autonomic nervous system, the endocrine glands and their products, the hormones, the reticulo-endothelial system, and the corpuscular components, *e.g.* of the blood, are involved (*c*). The electrolytes rank foremost in the regulating system but substances like the blood sugar, urea, vitamins and other bio-catalyzers such as enzymes also play an important part. It is evident that the mode of action of microelements mentioned before implicates an important interference with biochemical regulation.

In the scheme of the regulating system that I have published earlier (*c*), attention is focused on two microelements containing hormones, the thyroid hormone (I) and insulin (Zn), which exert a powerful action in relation to the sympathetic and the para-sympathetic nervous systems, respectively. Enzymes such as arginase (Mn), carbonic anhydrase (Zn), respiratory enzymes (Fe), coeruloplasmin and related substances (Cu) and xanthine oxydase (Mo), which all contain microelements in their molecules, as well as the numerous enzymes that are activated by microelements, contribute essentially to the biochemical balance in the animal body.

The whole problem should be tackled experimentally and in a more systematical way than was hitherto effected, from two angles. First of all attention has to be directed on shortages and excesses of indispensable microelements and secondly on non-essential microelements such as selenium, lead, mercury, baryum, arsenic, bromine and others which are incidentally present in the food, particularly those microelements exerting an influence on nervous tissue. In all these cases we may expect to be confronted with features of intoxication in the animal, either originating from a direct action of microelements on certain vital tissues (*e.g.* nervous tissue) or from metabolites present in excessive amounts in the animal tissues, due to an abnormal course of cell metabolism.

For the time being our knowledge with regard to the apprehension of the role of microelements in biochemical regulation is very poor. Yet a few observations suggesting the intricacy of these problems are to be mentioned.

It has been reported that the amount of zinc in foodstuffs is positively correlated with that of thiamine and therefore suggested that zinc deficiency may be a factor in the polyneuritis syndrome (10).

Manganese would play a part in the oxidation of monosaccharides to ascorbic acid and therefore be particularly important for animals that are independent of the intake of vitamin C (11).

Finally my co-worker S. T. Hofstra, starting from the analogy of the clinical features of copper deficiency in cattle and the symptoms of a shortage of B-vitamins, ascribes to copper a function in the synthesis of these vitamins in the rumen (12).

Until the results of further experiments are available, I would consider the facts revealed as a working hypothesis.

### *Microelements metabolism in health and disease*

Data so far available give rise to the assumption that in most species including man microelements metabolism seems to be regulated quite economically. Apparently these species make the most of a limited quantity of microelements in the food and symptoms of deficiency only develop under extremely poor conditions so far as the intake of microelements is concerned.

On the other hand, other species behave differently. Obviously their metabolism of microelements is based on waste. Absorption and accumulation seem to be poor, and excretion may be considerable. This holds, for instance, for ruminants-bovines and sheep, so far as copper is regarded.

In principle, the action of microelements on enzyme systems is not restricted to the enzymes in the animal tissues. It may be extended to the enzymes active in microorganisms living in symbiosis with mammals. In herbivora and particularly in ruminants the action of microelements on the metabolic processes of microbes seem to rank foremost, as compared with man and most other species of mammals.

Apparently in ruminants with their complicated gastro-intestinal system, the regulation of the optimal concentrations of certain microelements essential for normal life is more easily disturbed than in mammals with a simple gastro-intestinal tract. In agreement with this view, it has been shown that, in contrast with other mammals, in ruminants shortages of minerals, *e.g.* copper occur, although a real deficiency in the food is absent in most cases. This may be termed a 'conditioned deficiency' and it might suggests a mutual influence of the absorption and the metabolism of copper and other food constituents, in addition to the special action on the microbes in the forestomachs. Balance trials with cattle similar to those carried out in man with regard to copper (18) are progressing in my laboratory.

For the time being I am unable to present a complete outline, in this respect. However, a rough comparison between man and cattle clearly demonstrates the great differences existing between these species. By careful balance studies it has been possible to determine the dietary copper requirement of man (18). In the adult positive copper balance is maintained if the dietary intake is approximately 2 mg./day. According to our observations the daily intake of copper in a cow may be 200 mg. or more, and yet the copper balance may be negative.

Taking into account the difference in body weight between man and cattle, and the small loss of copper in the milk in cows, we may draw the conclusion that the daily intake of copper in cows may be about 10 times that in man per unit of weight.

A negative copper balance in cows particularly occurs during the summer season, when the animals are on pasture. In the winter time, when the animals are in the byre, the copper balance is positive, in general, even on a lower dietary intake of copper. It is my opinion that the imbalanced ration during the pasturing period, giving rise to increased intestinal movements, is mainly responsible for the conditioned copper deficiency observed in cattle.

I would express no opinion as to how far iron metabolism is equally economical in cattle as it is in human subjects. We know, however, that a deficiency in cobalt leads to a fatal malady in sheep and cattle (13) and it is reasonable to suppose that the cobalt activity in the rumen that may cure this condition is brought about by a number of vitamin $B_{12}$-like factors isolated by Kon and co-workers, and by other groups of workers, these factors acting on the rumen microbes (14).

From the standpoint of comparative biochemistry I think it to be instructive to compare the behaviour of mammals with a simple gastro-intestinal tract with that of ruminants particularly concerning the metabolism of copper and cobalt, the two elements which have been investigated more in detail in recent years. The problem of manganese which is also drawing much attention in recent time is still too vague to be thorougly commented at present.

The rat, rabbit, guinea pig (15) and calf (*f*, *g*) are born with a reserve store of copper in the liver which is drawn upon during the suckling period. Both the total amount and concentration of copper in the liver decrease during the suckling period. However, rats show an increase in total copper in the liver from birth onwards, even though the concentration decreases during suckling. This indicates that the rat receives a substantial amount of copper from the milk of the dam. This is in agreement with the finding that rat milk contains about ten times as much copper as cow's or human milk. As has been mentioned in table I the milk of the rat also contains much larger amounts of cobalt in the form of vitamin $B_{12}$ than the milk of other mammals including ruminants (9). The reason of these differences is obscure.

In the rat, rabbit and guinea pig the amount of manganese in the liver at birth is relatively low and the concentration of manganese varies relatively little during the life span. This observation apparently also holds true for man (16) and probably for bovines and horses (17).

The storage of copper in the liver of mammals including man is further mentioned in table II (*f*, *g*). In addition to other authors, my co-workers R. Bijkerk, S. T. Hofstra, J. van der Grift and K. J. Kruyt have essentially contributed to our knowledge in this respect.

Whereas the ratio between the lowest and the highest copper figures of the liver in normal mammals with a simple gastro-intestinal tract does not exceed 1 to 4, this ratio shows to be much wider, namely up to 1 to 28 in cattle. Newborn calves show an exception to this rule. The distribution of the copper values in newborn calves is shown to obey the Gauss law whereas in older cattle this law does not hold at all. This suggests a great variety of factors of internal and external origin which interfere with the process of copper storage in

TABLE II

*Copper content (p. p. m. dry matter basis) of the liver in normal mammals*

| Author | Number of samples | Species | Lowest | Mean | Highest | Range |
|---|---|---|---|---|---|---|
| Bijkerk | 6 | Man | 32 | 50 | 87 | 1 to 3 |
| Cartwright | 2 | Man | 24 | — | 60 | 1 to 2.5 |
| Bijkerk | 3 | Dog | 58 | 101 | 156 | 1 to 3 |
| Bijkerk | 37 | Horse | 13 | 18 | 32 | 1 to 2.5 |
| Bijkerk | 10 | Guinea Pig | 59 | 97 | 183 | 1 to 3 |
| Bijkerk | 10 | Rat | 14 | 24 | 55 | 1 to 4 |
| Cunningham | 12 | Pig | 12 | 19 | 48 | 1 to 4 |
| | | Cattle : | | | | |
| Hofstra | 15 | newborn | 70 | 174 | 298 | 1 to 4 |
| Cunningham | 41 | newborn | 143 | 381 | 655 | 1 to 4.5 |
| Hofstra | 10 | yearling | 8 | 86 | 226 | 1 to 28 |
| Cunningham | 6 | yearling | 16 | 67 | 137 | 1 to 8.5 |
| Hofstra | 10 | two years | 6 | 14 | 56 | 1 to 9 |
| Hofstra | 18 | adult | 3 | 17 | 58 | 1 to 19 |
| Bijkerk | 54 | adult | 5 | 27 | 91 | 1 to 18 |
| Cunningham | 23 | adult | 23 | 200 | 409 | 1 to 18 |

the liver, as soon as the animals grow older. The critical epoch, so far as this change of behaviour of cattle is concerned, appears to be at the age of about five months (table III). The reason of this change is still obscure.

TABLE III

*Alteration of the range of copper in cattle liver in relation to age*

| Age | Liver copper (p.p.m. dry wt.) | | | Range (8 calves) |
|---|---|---|---|---|
| | Lowest | Mean | Highest | |
| 24 hours . . . | 166 | 318 | 473 | 1 to 3 |
| 1 month . . . | 90 | 171 | 260 | 1 to 3 |
| 2 months . . | 74 | 114 | 185 | 1 to 2.5 |
| 3 months . . | 55 | 126 | 203 | 1 to 4 |
| 4 months . . | 70 | 146 | 211 | 1 to 3 |
| 5 months . . | 62 | 150 | 322 | 1 to 5 |

About 50 % of the livers of apparently normal adult dairy cattle in the Netherlands have a copper content varying from 3 to 10 p.p.m., a level usually considered to indicate a condition of deficiency. Yet, the herbage is not found to be poor in copper.

In our laboratory the storage of copper in the liver in healthy and diseased condition has been systematically investigated in cattle and horses.

In cattle the copper content of the liver was found to be decreased in gastro-intestinal disorders, including worm infestation (strongylosis) and Johne's disease. Furthermore a fall in liver copper was noted in disorders of the liver and the lungs, and in infectious diseases including tuberculosis. Pregnancy, however, was not shown to affect the copper content of the liver.

In horses a decrease of liver copper was only observed in pregnancy, whereas in lameness, gastro-intestinal disorders, infectious diseases, and disorders of the lungs including emphysema and edema, an increase of liver copper was noted.

In human subjects the copper content of the liver was found to be lowered in nutritional anemia (18). It is increased in all other disorders such as Mediterranean anemia, hemochromatosis, cirrhosis of the liver, acute yellow liver atrophy, tuberculosis, carcinoma and severe chronic diseases accompanied by anemia.

From this summary follows that, unlike human subjects and horses, in cattle a depletion of the liver so far as copper is concerned occurs in all diseased conditions hitherto examined. On the other hand an accumulation of copper in the liver has not been recorded in diseased cattle as it was in man and horses.

Apparently in cattle, as contrasted with human subjects and horses, copper metabolism is regulated uneconomically. The same conclusion may be drawn from the fluctuations of blood copper. Any stimulus of internal and external origin, *e.g.* an imbalanced ration giving rise to increased intestinal movements, is shown to make the available copper reserve in cattle worse.

The role played by copper in the microbial processes in the rumen is still obscure. Meanwhile the first action to establish a relation, if any, between copper and the rumen microbes, active in the processes of pre-digestion, has been taken. So far cobalt is concerned, a similar investigations has revealed that the rumen flora is changed in cobalt deficiency (19).

*Interrelationships of one microelement with another and of microelements with macroelements and with other food constituents*

A knowledge of the interrelationships among nutrients in a diet is essential for an understanding of their quantitative requirements for the animals. Utilization of one nutrient may be profoundly affected by the presence or absence of another. For instance, under certain circumstances the toxicity of zinc in rats may be corrected by copper, the presence of both molybdenum and zinc in any diet may result in a significantly poorer growth than was caused by the addition of these elements separately (20). Selenium poisoning may be reduced by arsenic (7); molybdenum poisoning in cattle may be corrected by copper (12). Intravenously administered methionine prevented the toxicity of high doses of cobalt (21). There is less absorption of iron from the gastro-intestinal tract in rats deficient in copper than in rats supplied with copper (22).

These observations and many others reaffirm the finding that an abnormal condition of the animal may not reflect merely a low or a high level of a dietary essential, but an excess or a shortage of one or more other nutrients which interfere with the normal metabolism of the essential dietary constituent.

One of the most striking examples of this kind concerns the assimilation and storage of copper in sheep (23). It was found in Australia that the addition of ferrous sulphide to the diet lowered the expected copper accumulation in the liver by 75 %. Zinc given in an amount of 100 mg. a day had an effect which was significant at the 5 % level, but when added in smaller amounts which would be available to sheep grazing normal pastures, it had no effect on copper retention.

Molybdenum given in the form of ammonium molybdate was found to have a severily limiting effect, but this effect was only observed when the diet also contained a sufficient quantity of inorganic sulphate.

Although more information will be required before full quantitative relationship between these three factors can be expressed, the data already presented enable to draw the conclusion that sheep can be in copper balance at much lower copper intake than has been generally believed, probably 1 mg./day or less. The assumption is made that when either molybdenum or sulphate intake is at the lowest level known to occur in pastures, sheep can accumulate copper in the liver at a very high rate and suffer the hemolytic crisis of chronic copper poisoning.

The nature of the interrelationship of one microelement with another and with other food constituents is still imperfectly or not at all understood. It is my opinion that it is within the scope of the biochemists' and nutritionists' major duties to clear up the obscurity on this domain as soon as possible.

I am fully aware of the fact that in this hour I have asked more questions than I have been able to answer. My activity as a research worker for thirty years have confronted me with so many difficulties and obscurities that I welcome the opportunity to bring a few of them to your notice in the hope to contribute in this way to a fruitful exchange of ideas.

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# The isolation and proof of structure of the vasopressins and the synthesis of octapeptide amides with pressor-antidiuretic activity (*)

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(*Received* 17 *June* 1955)

In our studies on the hormones of the posterior pituitary gland, we were able to isolate with Livermore (1) the oxytocic principle (oxytocin) in highly purified form through the application of Craig's method of countercurrent distribution (2) to one of the oxytocic fractions obtained by the fractionation procedure introduced by Kamm and co-workers (3). By the criterion of its behavior on countercurrent distribution, our material appeared to be quite homogeneous (1). This work with Livermore was confirmed and extended by work with Pierce (4) and with Pierce and Gordon (5) and culminated in the isolation of the crystalline flavianate of oxytocin, the first crystalline derivative of this hormone. For these studies in our laboratory, both hog and beef posterior pituitary lobes were used, and no difference was detected in the behavior of the oxytocin derived from these two sources (5).

While this work on oxytocin was under way, we turned to the isolation of the principle responsible for the pressor activity (vasopressin) from one of the pressor fractions which we obtained from lyophilized beef posterior lobes by the fractionation procedure of Kamm and co-workers (3). Various methods of fractionation were explored, and the most promising results were obtained by countercurrent distribution between *n*-butyl alcohol and 0.1 M aqueous *p*-toluenesulfonic acid with Turner and Pierce (6). After distribution, the solutions in the tubes containing the major portion of the pressor activity were combined, and the *p*-toluenesulfonic acid was removed with Amberlite IR-4B resin. The solution contained approximately 500 units of pressor activity per milligram, but on lyophilization the resulting product assayed 400 units per milligram, indicating that some inactivation had occurred during the concentration.

Analysis of a hydrolysate of this highly purified preparation according to the starch column method of Moore and Stein (7) showed the presence of phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, ammonia, arginine and cystine. The amino acids were in a molar ratio to each other of approximately 1 : 1 with approximately 3 moles of ammonia for one mole of each of the amino acids in the hydrolysate. Traces of leucine and isoleucine were present in the hydrolysate. In terms of the 8 amino acids and ammonia, 91 per cent of the weight of the unhydrolyzed compound was accounted for; the recovery of nitrogen was of the same order of magnitude. Later, in work with Popenoe (8), a preparation of 600 units per milligram was obtained by application of the same general purification procedure, which on hydrolysis showed the same composition; but no leucine or *iso*leucine could be detected.

It is of considerable interest that 6 of the amino acids which had been found to be present in a highly purified oxytocin preparation (4, 9) were also present in the vasopressin preparation. Hydrolysates of both of the principles contained cystine, glycine, aspartic acid, proline, glutamic acid, and tyrosine, but in the vasopressin, phenylalanine and arginine were present instead of the leucine and isoleucine found in oxytocin.

In sharp contrast to the result with oxytocin, a vasopressin was isolated from hog posterior pituitary glands in work with Popenoe and Lawler (10) which differed markedly in its distribution characteristics from the vasopressin isolated from beef glands. When the hog material was analyzed for amino acids, it was found to contain 7 of the same amino acid residues as the beef material, but lysine occurred in place of the arginine. This vasopressin material which we had isolated from hog glands has been termed lysine-vasopressin and the vasopressin from beef glands, arginine-vasopressin.

It was known from early studies on the molecular size of these posterior pituitary hormones by diffusion methods that the two materials possessed approximately the same size. Therefore, it was a fair assumption that, since oxytocin was shown by experiment to have a molecular weight in the neighborhood of 1000 (4), we were also dealing in the case of vasopressin with a molecule of that size. It is felt that subsequent work has justified this assumption with respect to molecular size.

In studies with Cohn and Irving (11), the isoelectric point of vasopressin was shown to be 10.85. In later studies with Taylor and Kunkel (12), the isoelectric point was determined by zone electrophoresis on filter paper, starch, and glass beads, and again the value was

(*) Congress lecture given at the closing ceremony, 6 August 1955.

10.9. This isoelectric point is fully explainable on the basis of a free guanido grouping on the arginine residue and a free phenolic grouping on the tyrosine residue in the hormone. Thus, from this physical evidence it would appear that vasopressin does not possess a free carboxyl group.

In tackling the degradation of vasopressin for the determination of its structure, an attack was employed which was quite similar to the one we had utilized for oxytocin and in addition, this chemical attack was supplemented with enzymatic studies. The chemical degradation studies were carried out with Popenoe (13, 14) and the enzymatic degradation with Lawler (15).

In order to find out whether the cystine residue in vasopressin was part of a cyclic structure, a high potency arginine-vasopressin preparation was oxidized with performic acid, which would cleave the disulfide linkage with the formation of two sulfonic acid residues. After the reaction with performic acid, the reaction mixture was subjected to countercurrent distribution (13). The principal peak of the distribution pattern corresponded closely to the theoretical curve calculated for a homogeneous substance. The material in this peak, moreover, had the same amino acid composition as vasopressin, except that in place of one residue of cystine there were two residues of cysteic acid, which would be expected if the oxidation had involved the disulfide linkage of the vasopressin molecule.

The apparent homogeneity of the performic acid-oxidized vasopressin was tested further by subjecting the material to two-dimensional paper chromatography and to column chromatography on the anion exchange resin Dowex-2. The product appeared to behave as a homogeneous substance in both systems. Thus it appeared from the oxidative data that the cystine residue in vasopressin was present in some type of cyclic structure.

Sanger's technique for the determination of the free amino groups of polypeptides and proteins (16) was applied to both vasopressin and the performic acid-oxidized vasopressin derived from it. When a high potency vasopressin preparation was treated with dinitrofluorobenzene, analysis of a hydrolysate by starch column chromatography showed that 6 of the original 8 amino acids remained in approximately equimolar amounts. The tyrosine had been replaced by O-dinitrophenyltyrosine and the amount of cystine in the hydrolysate after removal of any dinitrophenyl derivative was reduced considerably. It appeared that the alpha amino groups of phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and arginine were not free to react with dinitrofluorobenzene. Cystine was therefore suspected of possessing at least one free amino group.

The reaction of oxidized vasopressin with dinitrofluorobenzene was therefore studied. The same 6 amino acids which were not affected in the vasopressin reaction remained in approximately equimolar amounts in the hydrolysate of performic acid-oxidized vasopressin which had been treated with the reagent. Tyrosine was again replaced by O-dinitrophenyltyrosine and the amount of cysteic acid present was reduced from two to one mole. This was taken to indicate that one of the amino groups on the cystine residue in vasopressin was free.

These results on vasopressin were similar to those obtained on oxytocin, which also yielded one component on oxidation of the disulfide bond with performic acid (17) and showed essentially the same effects upon treatment with dinitrofluorobenzene (18, 19).

Since results derived from the studies on oxytocin and vasopressin had so far paralleled each other so closely in the degradative reactions studied, it seemed probable that helpful data might be derived from a study of the action of bromine water on performic acid-oxidized vasopressin as it had on oxytocin. This reagent had cleaved oxidized oxytocin into two fragments, one a dipeptide which was identified as β-sulfoalanyldibromotyrosine, and the other a heptapeptide containing cysteic acid along with the 6 remaining amino acids of the oxytocin molecule (19, 20). It had also been shown that the *iso*leucine residue in the heptapeptide contained a free amino group.

Treatment of performic acid-oxidized vasopressin with bromine water did bring about fragmentation of the molecule (13). One product of the reaction could readily be isolated by paper chromatography, and analysis of a hydrolysate of this product by chromatography on starch showed the presence of 7 amino acids, phenylalanine, proline, glutamic acid, aspartic acid, glycine, arginine, and cysteic acid in approximately equimolar amounts. This fragment apparently corresponds to the large fragment obtained by bromination of performic acid-oxidized oxytocin, with leucine and *iso*leucine replaced by arginine and phenylalanine. When this fragment derived from vasopressin was treated with dinitrofluorobenzene, only the amount of phenylalanine in the hydrolysate was significantly reduced, the other amino acids remaining in molar ratios to each other of approximately 1 : 1. It thus appeared that phenylalanine occupied a terminal position in the heptapeptide fragment from vasopressin which corresponded to the position of the *iso*leucine residue in the fragment from performic acid-oxidized oxytocin.

Production of this 'large fragment' by treatment of performic acid-oxidized vasopressin with bromine water involved the loss of one mole of cysteic acid and one mole of dibromotyrosine. By analogy with oxytocin, a 'small fragment', β-sulfoalanyldibromotyrosine, might be expected as one of the products of the reaction. However, a pure fragment of this nature was not found on the chromatograms. It was evident that considerable decomposition took place along with the fragmentation in the case of the bromine water treatment of performic acid-oxidized vasopressin.

Since, in performic acid-oxidized vasopressin, the only free amino group found by treatment with dinitrofluorobenzene was on one of the two residues of cysteic acid, and since the single residue of cysteic acid in the large fragment did not bear a free amino group, it seemed probable that the residue of cysteic acid which was terminal in the performic acid-oxidized vasopressin was the same residue which was removed by treatment with bromine water (13).

The performic acid-oxidized vasopressin was also submitted to the Edman degradative procedure (21), and it was found that cysteic acid and tyrosine were lost in steps 1 and 2, respectively (13). Thus it could be inferred

that the sequence at the end of the molecule adjacent to the free amino group in performic acid-oxidized vasopressin is β-sulfoalanyltyrosyl. During the treatment with bromine water, the amino group of phenylalanine is freed at the same time that the β-sulfoalanyldibromotyrosyl group is eliminated, which indicates that the tyrosyl residue in performic acid-oxidized vasopressin is linked to the phenylalanyl residue.

On the basis that no change other than the cleavage of the disulfide bond took place on treatment of vasopressin with performic acid, it seemed probable that in vasopressin the amino group on one half of the cystine residue is free, that the carboxyl group on that half of the cystine residue is linked to the amino group of tyrosine, and that tyrosine is in turn linked to the amino group of phenylalanine, the disulfide bond forming a part of a cyclic structure.

Partial hydrolysis studies were then carried out on the oxidized vasopressin in order to find out the sequence of the remaining amino acids (14). The material was hydrolyzed in a mixture of glacial acetic acid and concentrated hydrochloric acid at 37° C. for 7 days in a sealed tube under nitrogen. The contents of the peptides thus formed were determined by paper chromatography and are given in table I. These results, taken along with

TABLE I

*Peptides from partial acid hydrolysis of performic acid oxidized vasopressin*

| Peptide | $R_f$ with butanol-acetic acid | $R_f$ with 75 % phenol | Amino acids found on hydrolysis |
|---|---|---|---|
| Basic + neutral fraction | 0.12 | 0.64 | Cysteic-[Pro, Arg, Gly] |
| Cysteic acid fraction | 0.31 | 0.18 | [Cysteic, Tyr, Phe] |
| | 0.06 | 0.05 | [Asp, Cysteic] |
| | 0.05 | 0.03 | [Asp, Cysteic] |
| | 0.02 | 0.04 | [Glu, Asp, Cysteic] |
| | 0.08 | 0.08 | [Glu, Asp, Cysteic] |
| Carboxyl-acidic fraction | 0.53 | 0.64 | [Phe, Glu] |
| | 0.37 | 0.37 | [Phe, Glu, Asp] |
| | 0.18 | 0.10 | [Glu, Asp] |

the results already discussed on oxidation and Edman degradation, enable the following sequence of amino acids to be postulated for performic acid-oxidized vasopressin (14) :

Cysteic-Tyr-Phe-Glu-Asp-Cysteic-(Pro,Arg,Gly)

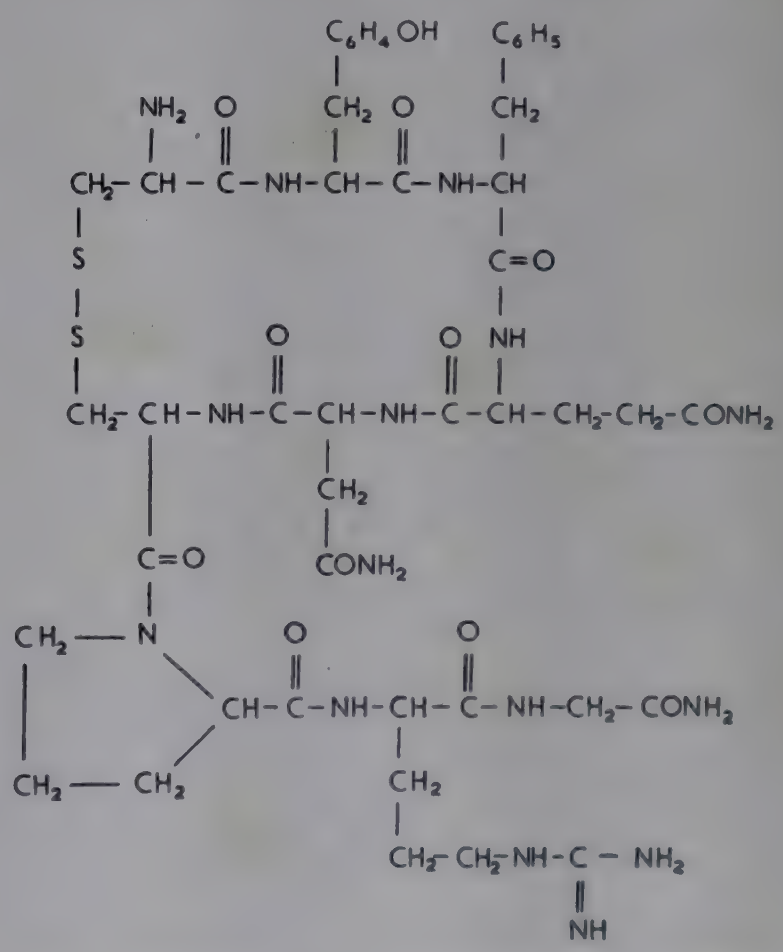
None of the peptides isolated from the partial hydrolysis shed any light on the sequence of the (Pro,Arg,Gly) end of the chain. Again, extending this sequence to vasopressin itself, the sequence of amino acids in arginine-vasopressin would be :

CyS-Tyr-Phe-Glu-Asp-CyS-(Pro,Arg,Gly)

While these degradative studies just discussed were being carried out, the action of enzymes on vasopressin was investigated. Through enzymatic cleavage of vasopressin with trypsin, it was possible to show that glycine was in the terminal position in vasopressin and that arginine occupied the penultimate position (22). After incubation with the enzyme, glycinamide was split off and another fraction containing the remaining amino acids found in vasopressin hydrolysates was obtained. The two fractions were separated and identified by two-dimensional paper chromatography. The crystalline enzyme from *Aspergillus oryzae* also released glycinamide from arginine-vasopressin (22).

Countercurrent distribution was also applied to the trypsin hydrolysate of arginine-vasopressin and again it was possible to identify the two components which had been obtained by paper chromatography. Incubation of the large fragment, which contained the 7 amino acids other than glycine which are present in hydrolysates of vasopressin, with arginase resulted in the liberation of some urea, whereas arginase has no action on the intact hormone under the same conditions. Since only those peptide bonds involving the carboxyl group of lysine or arginine are known to be hydrolyzed by trypsin, the liberation of glycinamide by trypsin from vasopressin indicates the sequence arginylglycinamide in the hormone.

With this information, together with all the results from oxidation, bromine water cleavage, Edman degradation, and partial hydrolysis, it was possible to postulate the following structure for arginine-vasopressin (22).



When lysine-vasopressin was subjected to hydrolysis with trypsin, it was also found to yield glycinamide (22). In view of the similarity in behavior of the two vasopres-

sins, it would seem logical that the proposed structure for arginine-vasopressin represents also that of lysine-vasopressin, with lysine replacing the arginine.

An attack upon the structure of arginine-vasopressin was also undertaken in Fromageot's laboratory in Paris. Although they had earlier come to the conclusion (23) that the sequence Pro-Arg-Gly-Glu occurred in the hormone, their later work indicated a Pro-Arg-Gly residue at the end of the hormone, and in fact, their work led to the postulation independently of a structure for arginine-vasopressin which was in agreement with the structure suggested above (24).

The postulations of the structure of vasopressin were based on a number of assumptions : firstly, that the 8 amino acids and 3 molecules of ammonia accounted for the composition of the hormone and that no small fragment not reacting with ninhydrin had been missed; secondly, that the structure contained only ordinary peptide linkages; thirdly, that the ammonia in the hydrolysate was derived from amide linkages in the hormone; fourthly, that no rearrangement had occurred during performic acid oxidation or in the partial hydrolysis; and fifthly, that glutamine and asparagine were present in the hormone rather than their isomers (*). Therefore, we felt that synthesis of the hormone was mandatory before any unequivocal conclusion could be reached with regard to the structure.

Before undertaking a discussion of the synthetical studies, it might be well to discuss the biological properties of the purified vasopressin, and it might be helpful to present likewise the biological properties of oxytocin.

The official method of the United States Pharmacopoeia for the assay of oxytocic fractions of the pituitary is based on their ability to lower the blood pressure of the chicken. The unitage of oxytocic fractions of the pituitary when measured by this method is the same as when the unitage is determined against the standard powder by their ability to contract the isolated rat or guinea pig uterus. The standard powder has been assigned the potency of 2 units per mg. Our highly purified oxytocin has been assayed, both in our laboratory and in the laboratory of Professor van Dyke at the College of Physicians and Surgeons of Columbia Univeristy, by both the avian depressor and the isolated rat uterus methods. We both found that either method of assay gave the same unitage of 450-500 units per mg. (8).

The purified oxytocin, in addition to the uterine-contracting activity and the ability to lower the blood pressure of the bird, is able to bring about the ejection of milk, and there is no doubt that it is the principal milk let-down factor of the pituitary, as shown in tests in sows by Whittlestone (25) and in rabbits by Cross and van Dyke (26). The isolation of the oxytocin has settled a problem that has been debated for many years as to whether the oxytocic principle was the milk let-down factor or whether there was still another independent hormone.

As assayed against the U. S. P. Standard Powder, the oxytocin contains approximately 500 units per mg. of milk-ejecting activity as well as 500 units mg. of oxytocic activity.

Through the collaboration of Professor Douglas and Drs. Nickerson and Bonsnes of our Department of Obstetrics and Gynecology, it was possible to test the milk-ejecting activity in recently parturient women. It was found that as little as one microgram when injected intravenously brought about milk ejection in 20 to 30 seconds (27). They also tested the ability of this purified oxytocin to induce labor and found it to be fully effective.

We thought at first that oxytocin was devoid of pressor and antidiuretic activity. However, we placed at van Dyke's disposal samples of our best oxytocin which were assayed by very refined techniques. They found 7 units of pressor and 3 units of antidiuretic activity per mg. These activities have been confirmed qualitatively and quantitatively with our synthetic oxytocin, so that these activities are inherent properties of the molecule (28).

With respect to the pharmacological effects of beef vasopressin, it possesses the full complement of pressor and antidiuretic effects, about 300 times as powerful as the U. S. P. Standard Powder, therefore 600 units per mg. In addition to these effects, the vasopressin possesses all of the effects of oxytocin, that is uterine-contracting, milk-ejecting, and avian vasodepressor activity, but to a lesser degree in each instance than oxytocin; about 1/20 for oxytocic, 1/5 for milk ejection, and 1/7 for avian depressor effects (8). Again, through the collaboration of our Department of Obstetrics and Gynecology, we have been able to test the milk-ejecting activity of vasopressin in the human. Vasopressin has a definite milk-ejecting effect and its potency is less than that of oxytocin.

We have convincing proof that these are inherent properties of vasopressin (8,15). As an antidiuretic substance, vasopressin represents one of the most potent pharmacological agents known. For example, Dr. van Dyke has shown that 5 one-hundred thousandths of a microgram of our material per kilo of body weight produces a marked antidiuretic response in a hydrated normal dog.

In considering the synthesis of the vasopressins, it was felt that the method of approach we were using for the synthesis of oxytocin might be employed. It may be recalled that the key intermediate we had selected for the synthesis of oxytocin was the following protected nonapeptide amide :

```
      Bz
CBzx-CyS-Tyr-Ileu-Glu(NH2)
                   |          Bz
                   |          |
                Asp(NH2)-CyS-Pro-Leu-Gly(NH2)
```

This method of approach was on the basis of the early work we had done on the reduction and reoxidation of oxytocin with Sealock in 1934 (29) in which we found that oxytocin could be reduced and reoxidized without appreciable inactivation. However, benzylation of the reduced form brought about inactivation. Similar exposure of the disulfide to benzylation did not produce inactivation.

(*) Degradative evidence for the presence of glutamine and asparagine in both oxytocin and vasopressin has been obtained by hydrolysis of the hormones with papain (42).

If our structure for oxytocin were correct, it would mean that the 20-membered ring could be opened and closed by reduction and reoxidation and that the reduced oxytocin would have the following linear structure :

```
H
CyS-Tyr-Ileu-Glu(NH2)
|                      H
|                      |
Asp(NH2)-CyS-Pro-Leu-Gly(NH2)
```

As we began to know more and more about oxytocin, there seemed to be no reason why benzylated reduced oxytocin should not be cleavable by sodium in liquid ammonia with regeneration of activity. In other words, if our structure for oxytocin were correct, we should be able to reduce and benzylate and get the S,S′-dibenzyl derivative of reduced oxytocin.

We decided to investigate this benzylation and debenzylation using highly purified oxytocin preparations with Gordon (30). Our best sample of oxytocin was treated with sodium and liquid ammonia followed by the addition of benzyl chloride to the liquid ammonia solution. From this reaction mixture we obtained a product which on amino acid analysis had the expected composition, containing 2 moles of S-benzyl-cysteine along with the other 7 amino acids present in oxytocin.

This material was biologically inactive. This dibenzyl derivative was then dissolved in liquid ammonia and metallic sodium was added. After getting rid of the ammonia, the product was dissolved in water and oxidized by passing air through the solution at a pH close to neutral, and we got a product which was biologically active. From a comparison of the physical, chemical, and biological properties of the starting and regenerated material, we were convinced that we had regenerated oxytocin from its S,S′-dibenzyl derivative. If we could synthesize the linear dibenzyl nonapeptide already shown with a suitable protecting group, such as the carbobenzoxy, on the amino group of one of the cysteine residues, we ought to be able to convert this protected nonapeptide by reduction and subsequent oxidation to oxytocin. This approach to the synthesis of oxytocin eventually proved to be successful (31, 32).

In the early work with Sealock, we had also found that vasopressin could be reduced and that the sulfhydryl form when injected exhibited biological activity. Reoxidation did not lead to appreciable inactivation. Furthermore, benzylation of the reduced form led to inactivation. It therefore seemed feasible that the same approach we were using for the synthesis of oxytocin might also be applicable to the synthesis of the vasopressins.

For the synthesis of lysine-vasopressin, an additional complication was present ; namely, the epsilon amino group of lysine. Since we had found in our earlier work with Behrens (33) that a tosyl group could be removed from an amino group with sodium in liquid ammonia, it appeared feasible to utilize a tosyl group as the covering group for the epsilon amino group of lysine during the various synthetic steps. Our proposed intermediate for the lysine-vasopressin was therefore :

```
  Bz
CBzx-CyS-Tyr-Phe-Glu(NH2)
|                     Bz    Tos
|                     |     |
Asp(NH2)-CyS-Pro-Lys-Gly(NH2)
```

The synthesis of lysine-vasopressin was undertaken in work with Popenoe and Roeske. It involved the preparation of a synthetic tetrapeptide, S-benzyl-L-cysteinyl-L-prolyl-tosyl-L-lysyl-glycinamide. The tetrapeptide was prepared by the reaction of ε-tosyl-α-carbobenzoxylysine with glycine ethyl ester and removal of the carbobenzoxy group from the resulting protected dipeptide with HBr in acetic acid. The tosyl-lysylglycine ethyl ester was then coupled with S-benzyl-N-carbobenzoxycysteinylproline and the carbobenzoxy group again removed with HBr in acetic acid. The resulting tosyl tetrapeptide ester was converted to the amide with ammonia in methanol.

This tetrapeptide amide was then condensed with the tripeptide, L-phenylalanyl-L-glutaminyl-L-asparagine (34) to form a heptapeptide which was condensed with the dipeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosine (35, 36) to form the synthetic intermediate for vasopressin, S-benzyl-N-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-tosyl-L-lysylglycinamide.

This protected nonapeptide was then reduced to the sulfhydryl nonapeptide with sodium in liquid ammonia and oxidized by aeration in aqueous solution to convert it to the cyclic octapeptide, as had been done in the last step of the synthesis of oxytocin. The crude product from this reaction possessed pressor activity, antidiuretic activity, and avian depressor activity in the ratios that were expected of it, although the yield of activity was low (22). The synthesis was repeated and confirmed, but again the yield of activity was very low. This synthesis is being reinvestigated, as well as other methods of approach to the compound. In the work thus far, the yield of active material has been considerably improved.

With the encouraging results on lysine-vasopressin, the synthesis of arginine-vasopressin was undertaken in work with Gish and later with Katsoyannis (37). In the case of arginine-vasopressin, the proposed synthetic intermediate was the nonapeptide corresponding to the intermediate shown for lysine-vasopressin, with arginine hydrobromide in place of the tosyl-lysine residue, and the required tetrapeptide for arginine-vasopressin was S-benzylcysteinylprolylarginylglycinamide monohydrobromide.

*p*-Nitrobenzyloxycarbonylarginylglycinamide was prepared by the procedure which had been worked out a year previous by Gish and Carpenter (38).

The *p*-nitrobenzyloxycarbonyl group was cleaved with hydrogen bromide in acetic acid, and the monohydrobromide of arginylglycinamide was condensed with *p*-nitrobenzyloxycarbonyl-S-benzyl-cysteinylproline by the pyrophosphite method (39) and the protected tetrapeptide was converted by HBr-acetic acid to the tetrapeptide amide required for the next step.

Instead of condensing the tetrapeptide amide with the tripeptide to give a heptapeptide and thence with carbobenzoxybenzylcysteinyltyrosine to the nonapeptide, we decided to condense the protected dipeptide with the

tripeptide phenylalanylglutaminylasparagine by the mixed anhydride method of Vaughan and Eichler (40) to give the protected pentapeptide.

This in turn was condensed with the arginine tetrapeptide amide monohydrobromide using tetraethyl pyrophosphite to give our desired nonapeptide amide intermediate (37).

The carbobenzoxy and benzyl groups were then cleaved with sodium in liquid ammonia and the resulting sulfhydryl nonapeptide was oxidized by aeration in aqueous solution at pH 6.7. The material from several runs gave us a total of 58 000 units of pressor activity. This synthetic product was purified by countercurrent distribution between *sec*-butyl alcohol and *p*-toluenesulfonic acid and then by electrophoresis in a pyridine-acetic acid buffer at pH 4.0 on a cellulose-supporting medium (41). The solution from the segment with the peak activity assayed 15 500 pressor units and upon lyophilization a powder weighing 37 mg. was obtained, which indicated a specific activity in solution of 400 units per mg. However, the lyophilization step of this highly active material caused partial inactivation of the material since the dried powder assayed 175 units per mg. The activity of this final synthetic product had the same position in countercurrent distribution, electrophoresis, and chromatography on partition and ion-exchange columns as the activity of natural arginine-vasopressin. It also possessed antidiuretic and avian vasodepressor activity, and in fact, the ratios between the pressor, antidiuretic, and avian vasodepressor activity were the same as those found for natural arginine-vasopressin (1 : 1 : 0.15) (37).

The synthetic product in all probability represents arginine-vasopressin, since the material possesses the biological activities associated with arginine-vasopressin in the proper ratios and since the activity in solution was as high as 400 units per mg. Moreover, the synthetic material showed the same behavior as natural arginine-vasopressin in countercurrent distribution, electrophoresis, and on partition and ion-exchange columns. Of course, the final proof of identify between the synthetic polypeptide and natural arginine-vasopressin must await the obtaining of the synthetic product in pure enough form to make an extensive and conclusive comparison of its chemical and physical properties with the natural hormone.

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# Chemistry and biochemistry of antimetabolites related to the purines

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(*Received* 4 *April* 1955)

The problem of definition of the subject matter of an essay, which frequently is troublesome, is particularly acute in dealing with « purine analogs ». One might consider any bicyclic aromatic system containing a six and a five membered ring as an analog of the purine system. Ring Index (1) listed 174 systems of this type when it was published, and further examples have been added since. One might hope to restrict the number of systems by adopting the criterion that one or more of the heteroatoms of the purine system be retained in the analogs. This device progressively diminishes the number of systems. However, even with the retention of 3 of the nitrogen atoms of the purine system a relatively large number of variants, in excess of 100, would easily be possible. Moreover, any such definition would neglect substances of possible interest : bicyclic systems with 2 six-membered rings, systems in which partial or complete reduction had taken place, and alterations in the functional groups of the naturally occurring purines.

Fundamentally, the justification for defining a substances as a 'purine analog' lies in the ability of the substance to evoke a biological response which in some way resembles or is antagonistic to the natural purines. This definition eliminates the greater number of possible analogs either by reason of their failure to produce a biological response of an acceptable character or simply because no biological testing has been reported.

For the purposes of this paper, it is proposed further to consider only substances related to the 'physiological purines' the two bases of the nucleic acids, adenine and guanine, and their deamination products, hypoxanthine and xanthine. Alterations of the functional groups of these compounds, with retention of the purine skeleton, have given rise to some of the more important antagonists of the natural purines.

Uric acid is considered to be a catabolic product beyond the point of no return to active participation in purine anabolism, as is indeed confirmed by studies with isotopically-labeled uric acid (2). The alkylxanthines and their analogues are also excluded from consideration by the nature of their biological activities.

A further restriction is imposed by the title of this symposium, namely that the advances discussed be 'recent'. For the purposes of this paper reliance is placed mainly on publications since 1950.

The purine system contains the elements of both the imidazole and pyrimidine rings, with two atoms shared in common at the point of fusion; it is in fact, imidazo-(4,5-*d*)-pyrimidine. This concept of purine as a hybrid with both imidazole and pyrimidine parentage is of some value in understanding the properties of derivatives of this system, and the synthetic approaches to specific purine derivatives. Total synthesis of the purine skeleton may proceed *via* a pyrimidine, methods most extensively studied by Traube (3, 4). Alternatively, a suitable imidazole may be synthesized first and the pyrimidine portion cyclized later (5, 6). Both approaches have been useful not only for the preparation of purines, but also with suitable modifications, for the synthesis of purine analogues, as will be discussed below.

## VARIANTS WITH INTACT PURINE SKELETON BUT UNNATURAL FUNCTIONAL GROUPS

Historically, the first approach to modified purines occurred through the transformation reactions, the study of which was initiated by Emil Fischer (7), and it is perhaps logical to begin a discussion of purine analogs with an examination of the biological significance of each of the functional groups and replaceable hydrogen atoms of the physiological purines. Studies along these lines have been reported *in extenso* by Hitchings, Elion and colleagues (8, 9, 10, 11); some of the more pertinent findings of these and later unpublished studies are presented in table I.

A number of modifications of the functional groups of the natural purines may lead to inhibitors. 2,6-Diaminopurine (table I, No. 2) was one of the earlier to receive attention. It is a potent inhibitor of *Lactobacillus casei* (12, 13) where it acts as an antagonist of adenine, as indeed it does in many other biological systems (14), despite the fact that in both the rat (15) and in bacteria (13, 16) it is freely converted into guanine or both guanine and adenine, respectively.

Alkylation of the amino groups of aminopurines diminishes their activity whether this be growth-promoting (compounds 1, 3, 4) or growth-inhibitory (compounds 2, 5, 6).

The introduction of an hydroxyl group in the 8-position also diminishes both stimulatory (10) and inhibitory activity (compound No. 7 *vs.* compound No. 2).

TABLE I

*Effects of purines on growth of* **Lactobacillus casei**

| Compound N° | Substituents | | | Conc. (mg./ml.) | % change in titre | |
|---|---|---|---|---|---|---|
| | 2 | 6 | 8 | | Medium OFA (*) | Medium OT (**) |
| 1 | H | $NH_2$ | H | 0.001 | +25 | +800 |
| 2 | $NH_2$ | $NH_2$ | H | 0.001 | —72 | 0 |
| 3 | H | $CH_3NH$ | H | 0.08 | +65 | +900 |
| 4 | H | $(CH_3)_2N$ | H | 0.1 | 0 | 0 |
| 5 | $CH_3NH$ | $NH_2$ | H | 0.1 | —78 | 0 |
| 6 | $(CH_3)_2N$ | $NH_2$ | H | 0.1 | 0 | 0 |
| 7 | $NH_2$ | $NH_2$ | OH | 0.08 | 0 | 0 |
| 8 | $NH_2$ | $NH_2$ | $NH_2$ | 0.004 | —67 | 0 |
| 9 | $NH_2$ | $NH_2$ | SH | 0.005 | —85 | 0 |
| 10 | $NH_2$ | OH | SH | 0.1 | —20 | 0 |
| 11 | H | SH | H | 0.1 | —84 | 0 |
| 12 | $NH_2$ | SH | H | 0.1 | —78 | 0 |
| 13 | H | $SCH_3$ | H | 0.1 | —10 | 0 |
| 14 | $NH_2$ | OH | (7-$CH_3$) | 0.1 | 0 | 0 |
| 15 | $NH_2$ | $NH_2$ | H | 0.02 | —61 | 0 |
| 16 | H | $NH_2$ | (9-$CH_3$) | 0.1 | 0 | 0 |
| 17 | $NH_2$ | O | (1-$CH_3$) | 0.1 | 0 | +700 |
| 18 | H | $CH_3$ | H | 0.005 | —75 | 0 |
| 19 | $NH_2$ | $CH_3$ | H | 0.1 | —90 | 0 |
| 20 | H | Cl | H | 0.1 | 0 | 0 |
| 21 | H | H | H | 0.01 | —91 | 0 |

(*) OFA : Basal medium (8) with 0.05 mμg. folic acid/ml.
(**) OT : Basal medium (8) with 1 μg. thymine/ml.
Control titre : OFA : 6.5 ml. 0.1 N/10 ml.;
OT : 0.8 ml. 0.1 N/10 ml.

Changes in structure which produce effects such as those described immediately above, are viewed as weakening the binding of the metabolite (or antimetabolite) to the cell receptor. Knowledge of such biological-chemical correlations is useful, at least in a negative way, in the problems connected with the design of new antimetabolites.

In contrast to the 8-hydroxyl derivatives, 8-amino and 8-mercaptopurines retain inhibitory activity (compounds 8 and 9) or attain mildly inhibitory activity (compound 10).

The replacement of hydroxyl by mercapto leads to inhibitory substances in the purine series (compounds 11 and 12) as indeed had been observed earlier with pyrimidines (17). 6-Mercaptopurine (18) and 2-amino-6-mercaptopurine are both of particular interest for their activities against neoplastic tissues (19, 20, 21, 22).

Alkylation of the imidazole ring greatly diminishes activity (compounds Nos. 14, 15 and 16). On the other hand the purine-1 position can be methylated with only moderate decrease in activity. It is of interest that 1-methylguanine resembles guanine in its ability to serve as a substrate for guanase (23) and that the corresponding methylthymine can replace thymine for growth (9).

The replacement of hydroxyl by methyl has in one case (No. 18) led to a very strong inhibitor, 6-methylpurine, which behaves as a purine antagonist with *Lactobacillus casei* and produces some dramatic toxicological effects (24). The 6-methyl analog of guanine (compound No. 19) however, is only a weak inhibitor, and it is not yet clear whether either of these substances is an antagonist of its hydroxyl isostere.

The replacement of hydroxyl by halogen, as in 6-chloropurine, has produced a substance which is inactive in the *Lactobacillus casei* system, but on other grounds appears similar to other 6-substituted purines (25). Finally, unsubstituted purine (26) itself (compound 21) is of considerable interest as an adenine antagonist, and the toxicity of the naturally-occurring purine riboside (27) may be attributable to this type of activity.

The majority of the purine derivatives mentioned above has been synthesized by the application of known methods or obtained through transformation reactions of a conventional nature. A few new transformation reactions and technical improvements in known reactions have been reported in the last few years.

The technique of chlorination in the purine series has been greatly improved by the addition of a tertiary amine to the phosphorylchloride (28). While in a few instances this led to unexpected results — xanthine, phosphorylchloride and triethylamine gave 2,6-bis-(diethylamino)-purine (29) — it has broadened the scope of chlorinations and made available derivatives (*e.g.* 6-chloropurine, 30) which previously had not been accessible.

A novel application of phosphorylchloride was described by Elion (31) in which 4-amino-5-benzamido-6-hydroxypyrimidines were simultaneously cyclized and chlorinated to yield 6-chloro-8-phenylpurines. Similarly 4-amino-5-formamido-6-hydroxypyrimidine has been found to yield 6-mercaptopurine on treatment with phosphorus pentasulfide (32).

The direct replacement of hydroxyl by mercapto through the action of phosphorus pentasulfide, used earlier for pyrimidines (33) has been applied successfully to the preparation of 6-mercaptopurine (34) and 2-amino-6-mercaptopurine (35) (thioguanine).

The synthesis of a variety of 6-aminopurines through the reaction of 6-methylmercaptopurine with amines proceeded smoothly (34). This is similar to the reaction of 2-hydroxy-8-methylmercaptopurine with methylamine (36) but in contrast to the failure of 2-methylmercaptoadenine to react with amines (37).

The reductive cleavage of mercapto and alkylmercapto by means of Raney nickel (38, 39) has recently been applied to the synthesis of purine from 6-mercaptopurine (40). The former also has been prepared by the catalytic reduction of 6-chloropurine (30).

The replacement of chloro groups by mercapto through reaction with thiourea and (if necessary) hydrolysis of the thiouronium derivative, which was introduced by Polonovski (41) has been applied to the synthesis of 6-mercaptopurine (30, 32).

A few new developments in the synthesis of purines from 4,5-diaminopyrimidines are worthy of mention. The cyclization of 4-amino-5-benzamidopyrimidine, by means of phosphorylchloride has been mentioned above (31). Bredereck (42) in several instances found benzamide and acetamide to give the 8-substituted purines by direct reaction with diaminopyrimidines. The cyclization of diaminopyrimidine sulfates merely by heating with formamide is apparently fairly

general (42, 43, 44) and represents a considerable advance over previous methods.

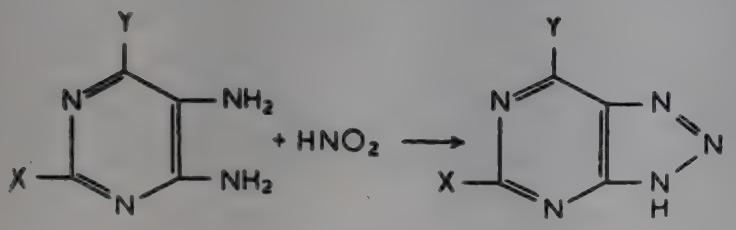
A new method of preparation of 8-aminopurines from 4-amino-5-N′-methylthioureidopyrimidines by treatment with mercuric oxide in the presence of an amine was recently reported (45). From the same laboratory there was reported the synthesis of 8-mercaptopurines through the action of carbon disulfide on 4,5-diaminopyrimidines (46).

## VARIANTS OF IMIDAZOLE MOIETY

The adaptations of the Traube-type synthesis from 4,5-diamino, 4- or 5-aminopyrimidines to the synthesis of purine analogues are many and varied. Reactions of this type lead to structures with the pyrimidine ring of the purine intact but with variants of the imidazole ring. The latter may be further subdivided on the basis of the retention of either or both the nitrogen atoms of the imidazole nucleus.

### *Compounds with 7-and 9-nitrogen atoms intact*

The *v*-triazolo-(*d*)-pyrimidines (1) are synthesized by what is essentially the Traube method for purines, from 4,5-diaminopyrimidines with the substitution of nitrous acid for the formic acid, leading to 8-aza- rather than normal purines :



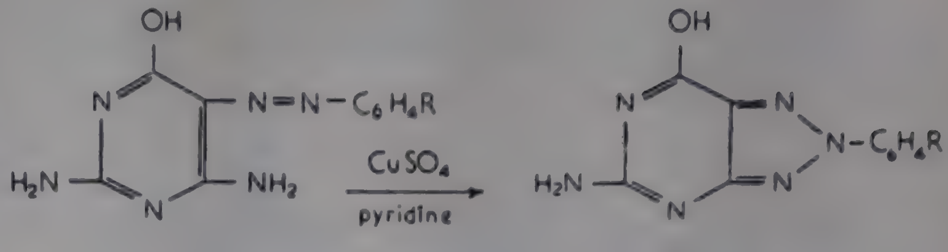
I

Equation 1

The first member of this series (**I**, X = H, Y = $CH_3$) was synthesized by Gabriel and Coleman (47) in 1901 ; however, interest in these compounds developed only after the synthesis of the analogs of the physiological purines (48). The guanine analog (**I**, X = $NH_2$, Y = OH) has attracted widespread attention and there exists concerning it a literature much too voluminous to review in detail in this place. It acts as a competitive antagonist of guanine (or guanosine) in many biological systems (48, 49, 50, 9). It inhibits the growth of several transplantable tumors (51, 52, 53) and plant virus infections (54, 55). It is incorporated into the nucleic acids of a variety of species (56, 57, 58) and is deaminated by the enzyme guanase (59) which has a high degree of specificity (23).

A considerable variety of *v*-triazolo-(*d*)-pyrimidines has been prepared essentially by the reaction of equation 1 above (60, 61, 62, 63, 67, 69) or by the transformation reactions of known derivatives (64). Some 2-phenyl-8-azapurine analogues of pteroic and folic acids (**II**, R = $CO_2H$, $CONHCH(CO_2H)CH_2CH_2CO_2H$) were synthesized by oxidation of 4-amino-5-phenylazopyrimidines (equation 2) (65).

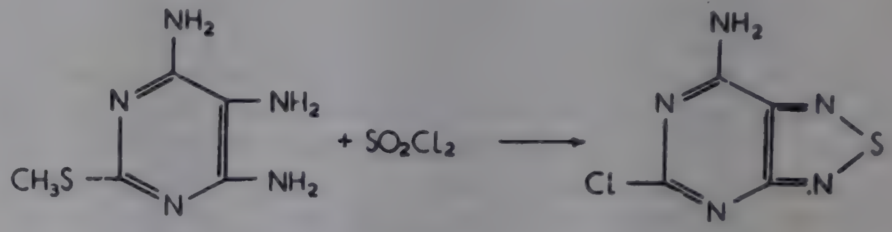
Only preliminary reports of the biological activities of these later derivatives have been published, and discussion of these should perhaps be deferred until more extensive data are available.



II

Equation 2

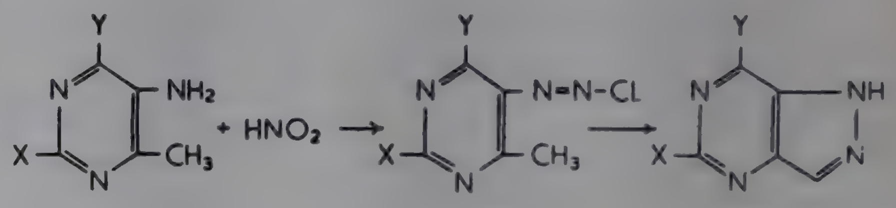
One further type of purine analog, prepared from a 4,5-diaminopyrimidine, has been reported (66) but this reaction was very limited in its application (equation 3).



Equation 3

### *Compounds with the 7-N atom intact*

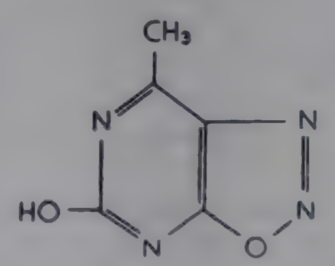
An extensive series of pyrazolo-(4,3-*d*)-pyrimidines (1,2,4,6-tetra azaindenes) has recently been reported by Rose (67) (equation 4). At first glance this appears to



III

Equation 4

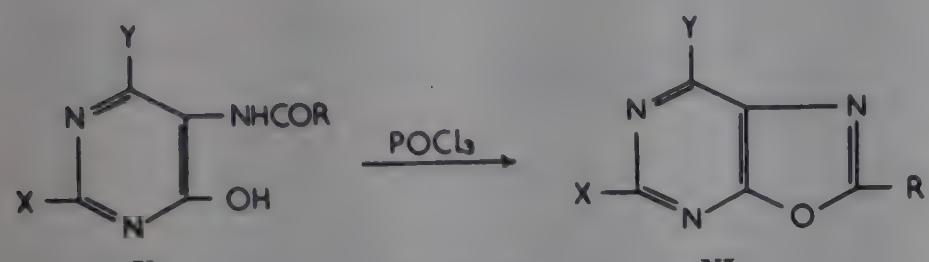
be the reaction of Behrend (68) for the preparation of *iso*xanthine (**III**, X = Y = OH). Rose, however, has brought forth evidence to indicate that Behrend's product in actuality anoxadiazole (IV) and that this is the



IV

preferred heterocycle formed by the action of nitrous acid on 5-amino-4-hydroxypyrimidines (67, 69). The biological activities of the pyrazolopyrimidines are of some interest, chiefly for the action of certain members (**III**, Y = $CH_3$, X = $HN_2$) on tubercular infections in mice (70).

A series of oxazolo-(5,4-*d*)-pyrimidines (71) was prepared from 5-amido-4-hydroxypyrimidines by the reaction shown in equation 5.

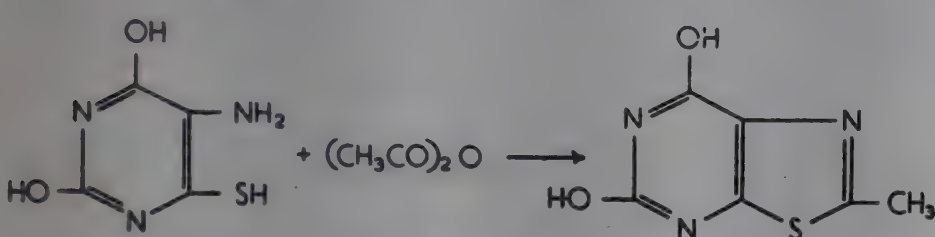


V VI

Equation 5

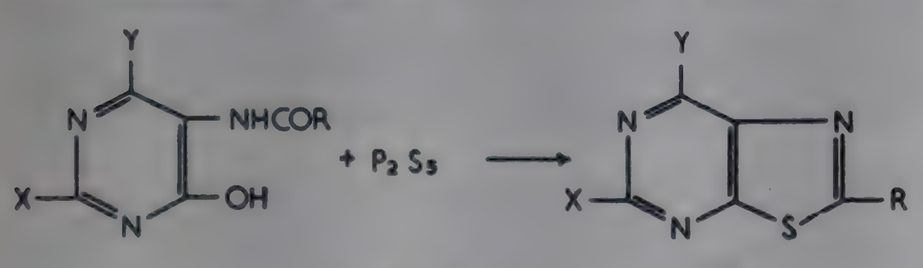
A point of some interest was that with 5-amido-4-amino-6-hydroxypyrimidines (**V**, Y = $NH_2$) cyclization to the purine was preferred as mentioned above (31); however, the proportion of oxazolopyrimidine could be increased markedly, although the purine was still the major product, through the addition of water to the phosphoryl chloride reagent. To date these products are of only slight biological interest.

Several workers have reported the synthesis of thiazolo-(4,5-*d*)-pyrimidines (**VII**). The first members of this series were synthesized almost simultaneously by Fischer (72) and by Weidel (73) in 1895 from thiouramil and acetic anhydride followed by saponification of the acetyl groups (equation 6).



Equation 6

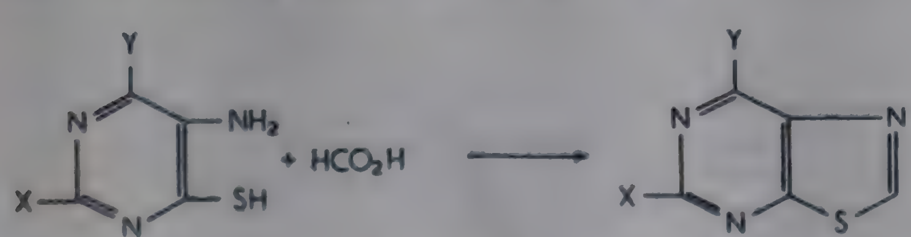
A novel method of cyclization, through the action of phosphorus pentasulfide on 5-amido-4-hydroxy-pyrimidine considerably extended the series (74) (equation 7).



VII

Equation 7

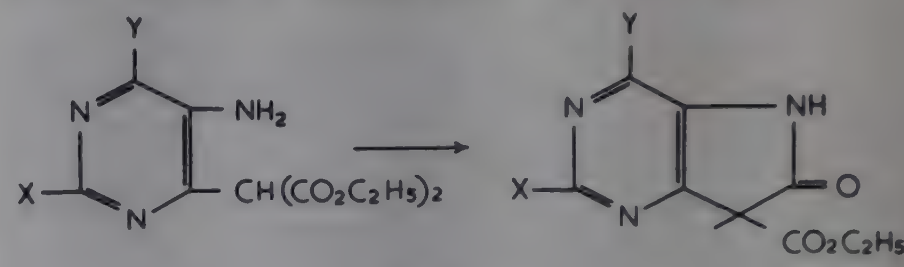
Another approach, *via* 5-amino-4-mercaptopyrimidines has also been reported (69) (equation 8).



Equation 8

The biological activities of these substances are of some interest, but not of a striking magnitude or character. The diamino derivatives, in common with the generality of 2,4-diaminopyrimidines and condensed systems containing the latter moiety are antagonists of folic acid in the growth of *Lactobacillus casei* (75).

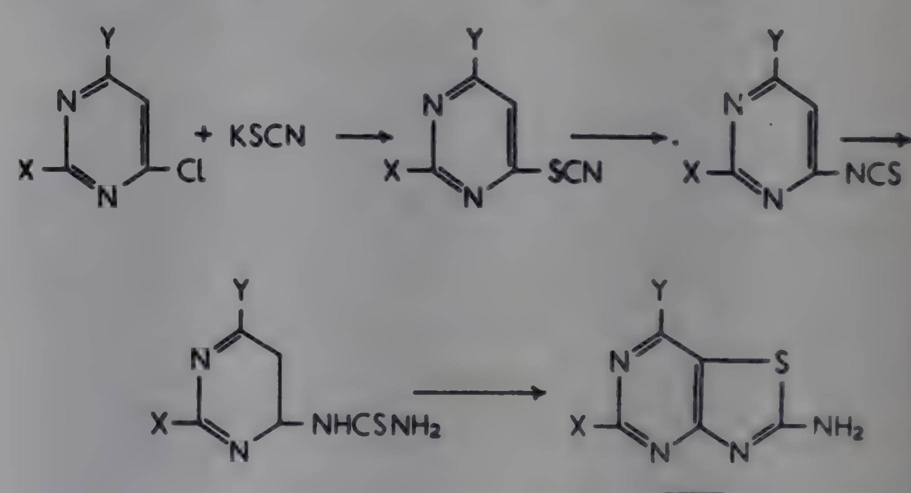
A few examples of pyrrolo-(3,2-*d*)-pyrimidines (1,4,6-triazaindenones) have been reported (67). These were formed by the cyclization shown in equation 9.



Equation 9

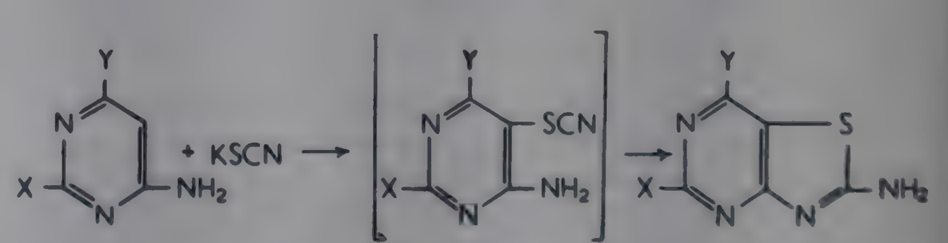
### *Compounds with the 9-N atom intact*

Purine analogs in which both the pyrimidine ring and the 9-nitrogen are retained are possibly of greater biochemical interest than the isomeric structures discussed immediately above. The replacement of the nitrogen atom in the 7-position of the purine skeleton by another atom or group, immediately places the synthetic approach outside the scope of the Traube concept. It is perhaps for this reason that methods of attack have been developed more slowly, and that only a few representatives of this type of analogs have been reported.



VIII

Equation 10



Equation 11

A series of thiazolo-(4,5-*d*)-pyrimidines was approached in two ways, chiefly because each method was restricted in its application to a limited numbers of functional groups in the pyrimidine moiety (76) (equation 10 and 11).

The 2-aminothiazolo-(4,5-*d*)-pyrimidines show some, but not a striking antagonism to the corresponding purines. Of particular interest is the 5-methylmercapto-7-amino derivative (**VIII**, X = $CH_3S$, Y = $NH_2$) which inhibits *Salmonella typhosa* as does 2-methylmercaptoadenine (77).

## VARIANTS OF THE PYRIMIDINE MOIETY

Benzimidazole, was the first imidazole derivative which was reported (78) to have the properties of a purine analog, on the basis of a competitive inhibition with adenine in the growth of several yeasts and bacteria. This substance continues to attract attention and is reported to act as an adenine antagonist in such diverse systems as the multiplication of polomyelitis virus in tissue culture (79) and the development of the frog embryo (80). These observations have prompted the synthesis of a number of benzimidazole derivatives (78, 81, 82) and similar analogs in the imidazopyridine and imidazopyrazine series. In general, substitution of the benzene nucleus, such as in 4-aminobenzimidazole, has failed to intensify the purine antagonism and the hydroxy- and amino-benzimidazoles appears to have more the character of phenols and anilines than of purines.

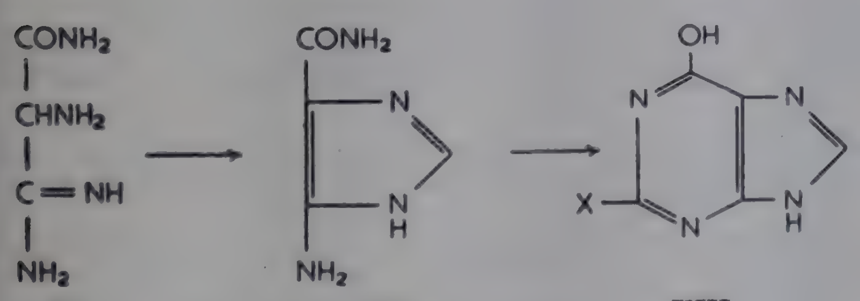
The synthesis of benzimidazoles and related compounds generally has been accomplished through cyclization of an *ortho*-diamine with a one-carbon reagent, as in equation 12.

IX X

Equation 12

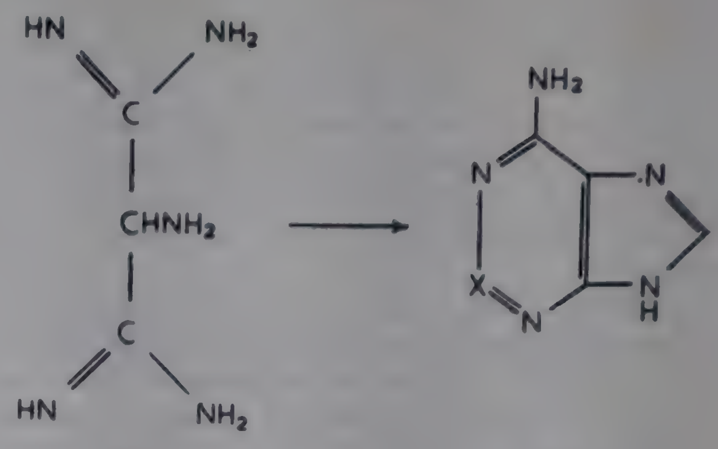
Korte (83) reported difficulty in the direct cyclization of 2,3-diaminopyridine (**IX**, X = CH, Y = N) which formed a diformyl intermediate which was cyclized only on heating with magnesium powder. However, Vaughan (84) in a similar series reports cyclization with formic acid, phosgene and thiophosgene. For the formation of imidazo-*b*-pyrazines (**X**, X = Y = N) triethyl*ortho*formate and urea were employed.

Adenine and hypoxanthine analogs in the 2-azapurine series have a considerable biological interest. Their synthesis derived from an earlier synthesis of the parent compounds *via* imidazole derivatives (85, 86) (equation 13, 14).



XI XII XIII

Equation 13



XIV XV

Equation 14

The methods employed constitute a new attack on the problem of the synthesis of imidazoles suitable for conversion to purines. Aminomalonamamidine (**XI**) was prepared from ethyl cyanoacetate and cyclized to hypoxanthine (**XIII**, X = H) and xanthine (**XIII**, X = OH) respectively. By starting with malononitrile the aminomalondiamidine (**XIV**) was attained and led to adenine (**XV**, X = CH) and *iso*guanine (**XV**, X = HOC). A number of variants were possible. The substitution of chloroformic for formic acid in the cyclization of the imidazole moiety led to an hydroxyl group in the imidazole-2 (purine-8) position (87). The substitution of nitrous for formic acid in the cyclization of the pyrimidine moiety led to the 2-aza-analogs of adenine (**XV**, X = N) and hypoxanthine (88).

These compounds proved to be antagonists of the purines in the growth of microorganisms and served as substrates and inhibitors of the action of xanthine oxidase (87). The adenine analog damages the cells of Sarcoma 180 in tissue culture (89). The approach to purines *via* acyclic intermediates is of particular interest in its resemblance to the biosynthetic mechanisms, which appears to proceed *via* glycinamide ribotide, formylglycinamide·ribotide and 4-amino-imidazolamide ribotide (90).

This review has not dealt with any of the structures in which both the imidazole and pyrimidine moieties differ from those of the purine skeleton. Few biological investigations of such structures have been reported, and many of the more obvious remain to be synthesized.

Finally, it should be mentioned that Mother Nature has not overlooked the possibilities of nucleic acid analogs as biological antagonists. Whether the sporadic occurrence in nature of *iso*guanine (91) and its riboside (92) has such significance remains to be seen. However, the inhibitory properties of nebularine (27), cordycepin (93) and puromycin (94) place them definitely in this category. Both cordycepin and puromycin contain abnormal sugars, the former contains adenine and the latter 6-dimethylaminopurine (18).

Two fundamental questions were raised at the outset of our own work 13 years ago. These were : 1) whether it would be possible to interfere with nucleic acid biosynthesis through the mediation of analogs of the heterocyclic bases of the nucleic acids, and 2) whether abnormal

bases could be incorporated into the nucleic acids. Both questions have now been answered unequivocally in the affirmative. The production of nucleic acids containing 8-azaguanine has been mentioned above. Similarly, 2,6-diaminopurine is incorporated into the nucleoside phosphates of the mouse (95) and the incorporation of 5-bromouracil, an antagonist of thymine (96) has been demonstrated for bacterial (97, 98) and virus (99) nucleoproteins.

It is obvious from studies on both the purines and some of the antagonistic substances of analogous systems that only a limited number of variants of the functional groups and replaceable hydrogen atoms are acceptable to the cell receptors of the generality of biological systems. The evaluation of many of the ring structures which resemble the purine structure is hampered because the desired functional substitutions have not been obtained. It is however, abundantly clear that considerable variation in response from organism to organism is exhibited by some of the structures, and this selectivity lends encouragement to the view that purine analogs may be potential chemotherapeutic agents (8, 9).

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# Purine nucleoside analogs as potential antimetabolites


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(*Received* 14 *July* 1955)

The immense amount of effort which has gone into studies of syntheses of purine analogs by both old and new methods, and into testing of their biological effects is indicated by Dr. Hitchings' comprehensive review. The fact that there are now a number of examples of purine analogs which inhibit biological systems is evidence of the actual and potential fruitfulness of this approach to the chemotherapy of disease. The magnitude of the total possible task, both chemical and biological, was accented by the limitations which Dr. Hitchings necessarily imposed upon the area of his discussions. It is also indicated by the fact that the investigators in the Chemotherapy Division of the Sloan-Kettering Institute have tested nearly two thousand pyrimidines and purines, relatively few of which are variations of the ring skeleton or are glycosyl substituted purines.

Desirable candidate antimetabolites must still be selected empirically, and the enormous choice possible can be limited only by a small amount of reasoning, for which Dr. Hitchings once coined the very apt term 'enlightened empiricism' (1). He has mentioned one obvious and universal guide, that of a preference for close structural analogs of the common natural purines, particularly adenine and guanine. To this should also

be added a preferential emphasis on analogs of any of the compounds which can be demonstrated to enter into anabolic reactions in biological systems (2). I would like to also stress that variants of any naturally occurring purine derivative deserve emphasis, regardless of how exotic the known locus of occurrence may be.

There is little I can add to what has been said regarding purine analogs, but would like to emphasize the next extension and complication of the general problem. This is the subject of the pharmacological and chemotherapeutic possibilities of the nucleosides of purines and their analogs, a subject which has hardly been explored. In most instances purines (or pyrimidines) are found in nature only as ribosyl derivatives; in the nucleic acids, in many of the coenzymes, or as the free mononucleotides. An increasing number of natural products which possess antibiotic or other biological activities are proving to be nucleosides or derivatives thereof.

Free purines are not known to play a role in normal anabolic reactions, but we now know that several purines can be rapidly anabolized into nucleotide derivatives (3), that some nucleosides can be phosphorylated to nucleotides (4, 5), and, perhaps more important, that nucleotides are probably the first purine derivatives arising by synthesis *de novo* (6). In several instances there is evidence which indicates that the mechanisms of action of purine analogs involve conversion to nucleotide derivatives, for instance, 2,6-diaminopurine (7) and 8-azaguanine (8), and that it is the metabolites of the purine analogs which may produce the biological effects by interference with the functions of coenzymes or nucleic acids.

The next few years must see not only the continuation of studies of purine analogs, but also more studies with larger molecules into which they might be anabolized. This will include not only applications of present methods to the preparation of new glycosyl derivatives of purines and their analogs, but also extensive studies of new synthetic approaches, and of the carbohydrate chemistry involved in obtaining the appropriate glycosyl derivatives. Synthesis and testing of nucleotides are, of course, also desirable goals but they present an even greater problem; despite recent extensive work on their synthesis (9, 10) the preparation of quantities sufficient for pharmacological testing is a most formidable task.

In some early metabolic studies we had the first evidence (11) that some ribosyl derivatives could have a metabolic fate different from that of the parent heterocycle and we began to devote some specific attention to nucleosides. Six years ago Dr. John Davoll came to our laboratory from that of Prof. Todd at Cambridge, and methods were developed (12, 13) for the synthesis of isotopically labeled nucleosides. These methods were also more suitable for the synthesis of the quantities of compounds which are needed for biological testing.

Certain of the purine nucleoside analogs so prepared (14) have yielded tissue specific inhibitory effects on tissues in culture (15), and in most cases the blocking by 'normal' nucleosides suggests that they generally produce their effects in an orthodox antimetabolite manner. Another study has demonstrated that appropriate analogs of a biologically active nucleoside may show a great enhancement of pharmacological activity. Several 2-substituted adenosines show a considerably greater vasodepressor activity than the parent adenosine (16); other alterations of the molecule destroy that activity; the 6-amino group and the 'normal' configuration of the glycosyl moiety are necessary. This same series of adenosines show effects on smooth muscle (guinea pig uterus, or rabbit intestine) (17), and the specific effect of inducing alterations in the glandular stomach of the rat which closely resemble clinical peptic ulcers (18).

The toxicity of ribosides of purines to the intact mammal is usually much less than that of the parent purine, perhaps because the nucleosides are frequently more adequately catabolized. However, the riboside of unsubstituted purine (19) is many times more toxic than the parent purine (20), and the extreme toxicity is accompanied by generalized tissue edema and hemorrhage. This naturally occurring (21) nucleoside is metabolically converted into the usual nucleic acid purines and into several acid-soluble nucleotides (22), and it is probable that one of these metabolites is responsible for the toxic manifestations. In this instance the high toxicity *in vivo* is obtained with the intact nucleoside, and not with its purine component. In tissue cultures *in vitro* similar toxic effects are obtained with either the riboside or, at much higher levels, with purine (23). An analog of purine riboside, 6-methylpurine riboside, is similarly somewhat more toxic than is the 6-methylpurine (20), and these examples emphasize the importance of testing the nucleosides as well as the purines.

The number of potent and specific biological activities which have been observed among the few nucleosides studied demonstrate the potential of this type of compound, and, despite the immense amount of effort which will be necessary, many more nucleosides must be investigated.

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# Incorporation of unnatural bases into nucleic acids


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(*Received* 10 *August* 1955)

Drs. Hitchings and Elion noted that one of the more interesting questions regarding purine and pyrimidine analogues is whether such compounds can replace normal bases in nucleic acids. This has now been established for several compounds and I should like to discuss this aspect of the subject. Dr. J. D. Smith will be describing some of our more recent results with 8-azaguanine later in the week (1). Rather than duplicate his remarks I shall present a very brief survey of the present position regarding incorporated analogues.

There are a number of statements in the literature that analogues have been incorporated into nucleic acids. Many of these are based only on the evidence that an isotopically labelled analogue was found in a nucleic acid preparation. This is no proof that the analogue was forming part of a polynucleotide chain since contamination with small amounts of the free base frequently occurs. In nucleic acids with a constant composition such as DNAs and virus RNAs base analysis may give strong evidence for incorporation. However, proof must come from the isolation and characterisation of the appropriate nucleotides of the analogue from the nucleic acid. Such evidence is available for two types of compound : the guanine analogue 8-azaguanine and the 5-halogenated uracils, which are analogues of thymine.

8-azaguanine is incorporated into the RNAs of many organisms when these are supplied with the compound. The proportion of guanine replaced varies widely in different systems (table I). *Bacillus cereus* is the only organism in which we detected incorporation of 8-azaguanine into DNA. Less than 1 % of the DNA guanine was replaced compared with 20-40 % for the RNA.

In several systems 8-azaadenine, 8-azahypoxanthine and 4-(5)-amino-1H,1,2,3,-triazole-5(4)-carboxyamide can give rise to 8-azaguanylic acid in the RNA. The question whether 8-azaadenine is incorporated as such has not yet been adequately investigated, but it seems highly probable that this will be found to occur in some systems at least.

TABLE I
*Incorporation of 8-azaguanine into various RNAs.*

| Source | % guanine replaced |
|---|---|
| Plant Sources : | |
| T. M. virus | 3 % |
| T. Y. M. virus | < 1 % |
| Pea embryos | < 1 % |
| Mouse Tissues : | |
| Spleen | |
| Liver | 0.5-1 % |
| Tumour (S 37) | |
| Bacteria | |
| *Bacterium coli* | 1-2 % |
| *Staph. aureus* | > 2 % |
| *Bacillus cereus* | 40 % |

Dunn and Smith (2) and Zamenhof and Griboff (3) have shown that 5-chlora, 5-bromo and 5-iodo uracils can replace thymine in *Bacterium coli* and $T_2$ virus DNAs. The amounts of normal base replaced may be large (table II).

There is some evidence for the incorporation of 2-thiouracil, 6-mercaptopurine and 2-azaadenine into nucleic acids, but further work is required with all these compounds.

All the analogues that are incorporated into nucleic acids are growth inhibitory for the organisms in which incorporation occurs. There are a number of lines of evidence suggesting that the growth inhibition may be due at least in large part to the production of non-functional nucleic acid. Here I shall mention only the most direct kind of evidence we have available at present.

TABLE II

*Incorporation of 5-halogenated uracils into DNAs*

| DNA from | Analogue | % of thymine replaced |
|---|---|---|
| *Bacterium coli B.* . . . . . | 5-bromouracil | 35 % |
| *Bacterium coli B.* . . . . . | 5-iodouracil | 19 % |
| Phage $T_2r$ . . . . . . . . . | 5-bromouracil | 79 % |
| Phage $T_2r$ . . . . . . . . . | 5-iodouracil | 48 % |

From the data of Dunn and Smith (2).

The infectivity of viruses containing analogues in their nucleic acids is reduced (table III). We would like to think that the non-infective virus particles are normal in all respects except that they contain a proportion of one of the normal bases in their nucleic acid replaced by an analogue, and that such nucleic acid is incapable of initiating the production of new virus particles.

TABLE III

*Infectivity of viruses containing analogues*

| Virus | Analogue | % normal base replaced | Infectivity as a % of normal virus |
|---|---|---|---|
| T. M. V. . | 8-azaguanine | 3 | 50 |
| T. Y. M. V. | 8-azaguanine | < 1 | 40 |
| $T_2$ . . . . | 5-bromouracil | 79 | 30 |

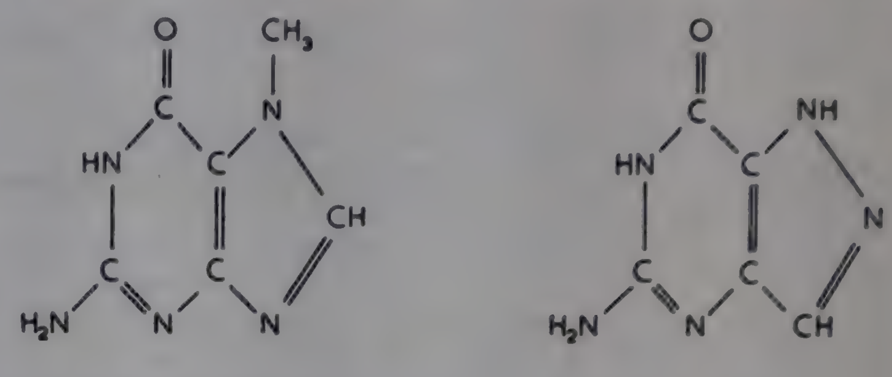
We have no clear idea at present as to what the significant chemical changes might be which could render the nucleic acid biologically ineffective. Further elucidation of this problem may give us a closer insight into nucleic acid function.

I should now like to consider some of the factors which may affect the incorporation of an analogue. The size and shape of 8-azaguanine and the 5-halogenated uracils will differ slightly from those of the corresponding normal base. It seems likely that for an analogue to be incorporated it should not differ too widely in size and shape from the normal base. However, at the present time we have no adequate basis for assessing the importance of these factors. For example, it seems idle to speculate about the sizes of the halogen atoms relative to the methyl group when we know that in one normal DNA the 5 position of a pyrimidine is substituted with hydroxymethylglucose.

Normal *Bacterium coli* DNA contains small amounts of 6-methylaminopurine (4). These workers showed that in *Bacterium coli* grown under conditions of thymine deficiency substantial amounts of thymine may be replaced by 6-methylaminopurine in the DNA. Such a substitution is impossible in the Watson and Crick model for DNA. This example clearly illustrates the present impossibility of making any firm predictions as to which bases may or may not be incorporated into nucleic acids.

The pKs of the functional groups in both 8-azaguanine and the 5-halogenated uracils are raised substantially compared with the normal bases. These changes may well be important in determ ning the effect of these compounds in nucleic acids.

Any analogue that is able to be built up into polynucleotides presumably must be able to form a glycosidic link of the correct configuration. On this basis we would consider that analogues such as those shown in figure 1 would not be incorporated into nucleic acids.



7-methylguanine

5-amino-7-hydroxy-1,2,4,6-tetraazaindene

FIG. 1. — Examples of analogues unlikely to be incorporated into nucleic acids through inability to form appropriate glycosidic linkage.

It is already quite evident that different organisms may vary widely in the way they deal with an analogue. As new types of compounds are developed they should be thoroughly tested in a wide variety of organisms. In particular systems an analogue may not be incorporated into nucleic acids for reasons that are essentially irrelevent to nucleic acid structure. Many analogues which are not incorporated when supplied as the base may be found to be incorporated when supplied as the appropriate nucleoside or nucleotide. There are well over 10 possible types of nucleotide for a given base. Further, with certain analogues (*e. g.*, 8-azaguanine) analogue precursors are known which might also be supplied as the nucleoside or nucleotides. Thus the number of possible forms in which an analogue could be supplied to an organism is quite large.

Finally I should like to make a few remarks about the distribution of analogues along the nucleic acid chain relative to the occurrence of the normal base. There is no reason to suppose that an analogue would replace the normal base at random. With 8-azaguanine in *Bacillus cereus* RNA we know that the distribution is not at random. Certain positions are relatively richer in 8-azaguanine than is the nucleic acid as a whole. Furthermore, we need not expect that the effect of a substitution by an analogue will have the same kind of biological effect in all positions. A given analogue may prevent function in one position or kind of position along the chain. It may alter function in other positions and may substitute completely for the normal base in others. However, there is at present no means of controlling the distribution of an analogue in the chains. As long as this is not possible it may be very difficult to detect changes due to an incorporated analogue that might be of a 'mutation' type.

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# The chemistry of mucopolysaccharides

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(*Received* 12 *April* 1955)

It would be too presumptuous an undertaking for me to try in the short time at my disposal to review the chemical work started 80 years ago in so complicated a field as the one of mucopolysaccharides. Excellent reviews (1, 2, 3, 4) have covered up to the present time a field in which the names of P. A. Levene, A. F. Charles, K. Meyer, E. Jorpes, H. Masamune, M. L. Wolfrom, M. Stacey have been and still are prominent. It is our intention to limit our discussion to the progress made in the last five years.

The definition of the class of mucopolysaccharides is still open to discussion and will be as long as the exact nature of the carbohydrate and of its linkage with proteins is not resolved. Then only will it be possible to base a classification on a sound chemical foundation. It is enough to point out that the content in proteins has been mentioned as a determining factor (2, 3) or the content in aminosugar (1). Like Blix (4) or Pigman and Goepp (5) we are inclined to limit considerably the group of substances included under that name and we will discuss only the compounds described by others as acidic mucopolysaccharides, *i.e.* the ones containing aminosugars and uronic acids, like hyaluronic acid, chondroitin sulfuric acid and the heparins.

However, it is evident that the methods used in the study of such a group of substances lend themselves to other classes of compounds, as for example the blood group polysaccharides and the bacterial polysaccharides.

### *Isolation*

Thirty years elapsed since the isolation of chondroitin sulfuric acid by Krunkenberg when, almost simultaneously, in 1916 the isolation of two new mucopolysaccharides was described, heparin by McLean and mucoitin sulfuric acid (probably a mixture) by Levene and Lope-Suarez. Eighteen years later, K. Meyer and his group described the isolation of hyaluronic acid from vitreous humor and were able in the following years to demonstrate its presence in many tissues as well as in bacteria. Hyaluronic acid did not contain O-sulfate groups and represented the simplest problem in the complex field of the chemistry of mucopolysaccharides.

Among the best characterized compounds recently isolated, we should mention β-heparin, described by Marbet and Winterstein (6). Like chondroitin sulfuric acid, it contains N-acetylgalactosamine, glucuronic acid and a sulfate-ester group, and possesses a weak but definite anticoagulant activity. The isolation and the study of corneal polysaccharides have been pursued by Woodin (7) and K. Meyer *et al.* (8). Their work denies the existence of a sulfate ester of hyaluronic acid, postulated by Meyer and Chaffee. Besides chondroitin sulfuric acid, K. Meyer *et al.* (8) were able to extract and characterize a polysaccharide formed of N.-acetylglucosamine, galactose and a sulfate ester group which was named keratosulfate. Such a polysaccharide had previously been isolated and characterized by Suzuki (9). In addition, a polysaccharide very similar to chondroitin sulfuric acid, but with a low content of sulfur was extracted and named chondroitin (10). K. Meyer and Rapport (11) have described the isolation from various tissues of three different chondroitin sulfuric acids. Whereas the chondroitin sulfuric acid (B) isolated from skin is definitely not identical with the one isolated from cartilage (A) (12), the differentiation between acids A and C, based on fractionation and on nearly identical physical and biological properties, is much less evident.

Various methods of isolation have been described recently. The difference lies mainly in the goal for which they have been worked out : complete extraction, fast extraction, extraction of a compound with minimum degradation, etc. The most important methods will be discussed in connection with the compounds to which they are applied. It is worthwhile to mention a general method of separation, on an analytical or preparative basis, based on electrophoresis in gel and first described by Gardell *et al.* (13). Without doubt, such methods, modified and amplified, will play a leading role in the future isolation and purification of new mucopolysaccharides.

### *Determination of compounds*

The qualitative and quantitative determination of the mucopolysaccharide components, *i.e.* aminosugars, uronic acid and ester groups, acetyl and sulfate, presents problems not satisfactorily solved up to now.

The characterization of aminosugars has been based on physical properties not easily evaluated (rotation, X-ray spectra) and on the preparation of well defined crystalline derivatives. However, the separation of nearly

equal amounts of glucosamine and galactosamine is practically impossible to be carried out by crystallization on small amounts of material. The use of paper chromatography has been hampered by the trailing of the substances in most solvent mixtures and by poor separation in others. A successful separation is obtained according to Yosizawa (14); unfortunately, a very long period of time for runs is necessary which causes large amounts of aminosugars to be degraded. In a brief communication in 1950, Gardell *et al.* (15) described a method for separating glucosamine and galactosamine, based on the well-known degradation of those aminosugars to the corresponding pentoses under the action of ninhydrin. The paper chromatographic separation was carried out on the resulting mixture of arabinose and lyxose. Dr. Stoffyn (16) in our laboratory developed the method and applied it to very small amounts of aminosugars (down to 2 µg.). It was successfully applied to a series of mucopolysaccharides, blood group polysaccharides, and intermediary products of the metabolism of aminosugars (17). It was possible to demonstrate the presence of 1 part of galactosamine in 20 parts of glucosamine in a sample of less than 100 µg. of hyaluronic acid containing 0.2 % of sulfur.

Such a procedure has been used not only for the characterization of aminosugars, but also to check the presence of small traces of another aminosugar in the α- and β-heparins and in chondroitin sulfuric acid of cartilage. Unfortunately, all attempts to estimate quantitatively the amounts of aminosugars with this procedure have failed up to now and the best method, the visual estimation of the spots, gives an approximation of 20 % with a limit of 5 µg. of one of the sugars in the presence of 100 to 150 µg. of the other.

Separation of the aminosugars as N-dinitrophenyl derivatives, by column adsorption (18) or paper chromatography (19) has been described. Because of the instability of the glucosamine derivative and the difficulty of its preparation in a pure state (20, 21) such a method has been superseded by the separation of the aminosugars as hydrochlorides on an acidic resin column described by Gardell (22). A similar procedure allowing separation and determination of the aminosugars in presence of hydrolysate of proteins has been recently reported (23). The drawback of such methods are the length of time and the amount of work necessary to analyze long series of effluent samples to determine the location of the components and their respective amounts, and the relatively large amount of material necessary to carry out a determination. Some of these disadvantages have been solved in the method of Leskowitz and Kabat (24). In their procedure, the aminosugars are reduced in aqueous solutions by sodium borohydride in the corresponding glycosaminitol from which the crystalline stable N-dinitrophenyl derivatives are prepared. A first separation eliminates the derivatives of amino acids and a second, more careful chromatography in presence of borate, separates the two sugars, which are determined subsequently by colorimetry.

Identification and determination of the uronic part of mucopolysaccharides are still the weak spots in their analytical study. The best method for identification seems to be the hydrolysis by sulfuric acid in presence of bromine as oxidant used successfully by Wolfrom and Rice in the study of heparin (25) and of mucoitin sulfuric acid (26). However, the yields in crystalline products are extremely small, because of the partial decarboxylation, during the hydrolysis of the very resistant glycuronidic linkage. Most new quantitative determinations are small improvements of two old methods, decarboxylation by strong acid with determination of the carbon dioxide evolved, and hydrolysis coupled with condensation with naphthoresorcinol or another reagent. The first method is very unspecific, whereas the second suffers from the difficult hydrolysis and gives poor yield. It seems that a method based on preliminary reduction of the uronic acid part to a hexose part, followed by hydrolysis and determination of the hexose liberated could be devised and would offer advantages over the methods already described.

The determination of acetyl groups was of special interest in the study of heparin and of the biological synthesis of hyaluronic acid. Most methods are based on acid or alkaline hydrolysis, followed by distillation and titration. As the procedure is unspecific, it has been found of value to corroborate the results obtained by a method based on oxydative scission (chromic acid) and such a determination is highly recommended for every new compound isolated. Present problems in the study of the sulfate group ask for a precise and rapid determination of the free sulfate in presence of the bound sulfate, applicable to micro amounts.

### *Chemical structure*

Identical methods as the one used for the determination of the structure of polysaccharides have been applied with varied degrees of success to the study of the chemical structure of mucopolysaccharides.

The simplest method consists in the condensation of the polysaccharides with substances known to react with preferential groups, like triphenylchloromethane with primary hydroxyl groups, or to prepare the tosyl derivatives and react them with sodium iodide, the tosyl group linked to the primary hydroxyl group being exchanged with iodine. Such reactions are not very successful with mucopolysaccharides, because of their insolubility in the medium commonly used for the condensation. In addition, the uronic part does not react, thus the information obtained is very poor.

Studies of the periodate oxidation of pentoses or hexoses containing polysaccharides have been well developed. Such reactions are readily applied to aminosugars derivatives and give reproducible results under carefully controlled conditions (27). Periodate oxidation of uronic acids is more difficult to interpret (28). Results obtained with periodate oxidation of mucopolysaccharides should be examined very critically and cannot lead to definite characterization of a structure. The presence of uronic acid, N-acetyl, sometimes sulfate groups, hinder the oxidation and consequently lessen the possibilities of interpretation. However, even with all its limitations, the periodate oxidation is still a useful method in the study of the structure of mucopolysaccharides and can be of great help in the screening of the extremely great amount of possible structures.

Most complete information is obtained by the methylation procedure, followed by hydrolysis and identification of the methylated fragments. It allows the determination of the positions of linkages between components, the nature of the component ring and the degree of branching of the polysaccharide. The stumbling block in the procedure is the carrying to completion of the methylation, without extensive degradation. Because of the resistance of the glucuronidic linkage, methanolysis leads generally only to the disaccharide without degradation of the uronic part. Sulfate groups are usually split during the methanolysis. Completion of the scission to monosaccharides without degradation can be accomplished only after the reduction of the glucuronide part into a glucoside part. The new reducing agents such as lithium aluminium hydride (29) and sodium borohydride (30) have been of great help in solving this problem. Identification of the methylated fragments is easy for the glucuronide part, as it is transformed by the reduction in a methylated glucose and most of the methylated glucoses have been synthesized (31). Three routes lie open for the identification of the methylated aminosugars. The transformation to a methylated osazone was discarded because of the difficulty in obtaining crystalline methylated osazone in small amounts and the small yield of the transformation. Degradation to the corresponding, methylated pentoses would have presented the problem of the synthesis of most of the methylated pentoses and a study to make the reaction quantitative for analytical purposes. Instead we chose to synthesize the various methylated aminosugars and to study their separation and identification on a very small scale. If we assume the sugars to be in the pyranose form, seven derivatives can be obtained for each sugar. Up to the present time, the synthesis of the monomethyl derivatives in positions 3 (32) and 6 (33), the dimethyl derivatives in positions 3, 4 (34), 4, 6 (35) and 3, 6 (36), and the 3, 4, 6-trimethyl derivative (37) of D-glucosamine have been described, whereas the preparation of 4-methylglucosamine is under way. In the D-galactosamine series, the synthesis of the monomethyl derivatives in positions 3 (38) and 4 (39), of the dimethyl derivative in positions 4, 6 (40), and of the 3, 4, 6-trimethyl derivative (41) have been described, whereas the synthesis of 6-methyl- and 3, 4-dimethylgalactosamine are under way. The limitations of the methylation procedure make it impossible to determine the configuration of the glycosidic linkages or the exact location of more than one substituent linked to a hydroxyl group. In a case like that it is necessary to carry out the methylation on partially degraded products, which leads to the last method, the study of the partially degraded products of mucopolysaccharides.

The degradation is carried out by chemical (generally hydrolysis by acid) or biochemical (action of enzyme) agents and leads to oligosaccharides, generally disaccharides, isolated in pure form, which are in turn studied with the classical means of carbohydrate chemistry : preparation of derivatives, periodate oxidation, methylation, transformation into compounds known or susceptible to synthesis. No general method can be applied and each mucopolysaccharide will require a different approach. For each linkage, a disaccharide would have to be isolated in pure form, which is a serious limitation of the method. Moreover the ester groups will be removed during the degradation with no chance of determining their respective positions. However, if a derivative still containing the glycosidic linkage can be synthesized, it is then possible to determine the anomeric configuration. An additional drawback of the enzymatic degradation is the possibility of biochemical synthesis or transglycosidification, and any compound isolated in small amounts could lead to incorrect assumptions.

It is likely that only a combination of the two last methods will give the best results in the determination of the configuration of mucopolysaccharides. However, the complexity of the problem (36 possibilities for chondroitin sulfuric acid, if both sugars are only in the pyranose form !) and the inability to use the periodate oxidation as a tool for specific degradation, due to the high degree of substitution, make impossible at the present time, a determination of the chemical structure of mucopolysaccharides with the high degree of certainty obtained in the glucose containing polysaccharides.

A review of the research done on the structure of various mucopolysaccharides will show the use made of the above methods.

### *Hyaluronic acid*

*Isolation and physical properties.* — Various methods have been described with the purpose of obtaining a prompt elimination of the proteins (42, 43), a very pure (44) or a very viscous (45) product. Those methods do not seem to improve on the previous ones described, for obtaining with a good yield and a minimum of degradation a product free of proteins and of chondroitin sulfate, in a few steps and on a large scale.

Studies on the physical state of hyaluronic acid have been reported recently in numerous publications : viscosity, osmometry, migration in the electrophoretic field, dielectric dispersion, light scattering, etc. They confirm and elaborate the figure described in preceding works. Discussion of their results would be outside the scope of this report. It is sufficient to mention that the particle of pure hyaluronic acid from umbilical cord has a minimum molecular weight of about $10^6$ (46, 47), behaves like a somewhat rigid coil and migrates in the electric field with a speed of about $14 \times 10^{-5} cm^2 \times sec.^{-1} \times volt^{-1}$ at pH 8.3, $\mu$ 0.1 (44).

*Chemical structure.* — Examination by infra-red spectroscopy of samples of hyaluronic acids extracted from various tissues did not show significant differences (48). It confirms the presence of the various groups already determined. A study of the carboxylic group (49) has shown that it was not present in bound form and disproved the assumption of anhydride groups in hyaluronic acid (50). The preparation of a trityl derivative, in low yield, because of the unfavorable conditions of the reaction, showed that position 6 of the glucosamine moiety was not linked. Because of the same unfavorable conditions, it was not possible to acetylate completely the hydroxyl groups or to prepare a *p*-toluenesulfonyl derivative (51).

Various laboratories have described periodate oxidation of hyaluronic acid (52, 53, 54, 55). The amount of oxidant used is very small and is probably due to the oxidation of the chain extremities. The only structure fitting such a result is a straight chain with glucosaminidic linkage in positions 1-3 and glucuronidic linkage in positions 1-3 or 1-4. A choice between those two alternatives was made in preparing a partially deacetylated hyaluronic acid. Periodate oxidation of chitosan (deacetylated chitin) in which the linkage is in position 4, released 0.7 mole of ammonia for each glucosamine unit, in agreement with the theory (56). Periodate oxidation of partially deacetylated hyaluronic acid did not release any ammonia. Thus a glucuronidic linkage in position 1-3 was postulated (52). The value of such a determination is limited by the fact that the uptake of oxidant by hyaluronic acid is negligible only under very controlled conditions and by the fact that the partially deacetylated hyaluronic acid was prepared in a poor yield.

The methylation of hyaluronic acid has been accomphished in four laboratories (57, 53, 54, 58). Methylation at elevated temperature with dimethylsulfate and sodium hydroxide, followed by treatment with methyl iodide and silver oxide (57) led to degraded products, from which only traces of crystalline substances could be isolated after methanolysis. Methylation in the cold with dimethyl sulfate and sodium hydroxide, followed by treatment of the salt-free acid with diazomethane afforded a product with a methoxyl content of 30.4 % (theoret. 34.5 %) in a 70 % yield. No chemical degradation had taken place and the product still showed a very strongly negative rotation (58). Scission of methylated methyl hyaluronate was carried out by methanolysis. K. H. Meyer, Fellig and Fischer (53) and Blix (54) did not analyze the resulting mixture from the scission, and carried out directly the periodate oxidation on it, assuming that it contained monosaccharides. As it has been demonstrated later that the degradation stopped at the disaccharide level (59), their conclusions based on the incorrect assumption of a mixture of monosaccharides, cannot be accepted.

Methanolysis of methylated methyl hyaluronate, followed by acetylation and chromatographic separation gave in a 60 % yield a crystalline methyl 3-0-(methyl 3(?)-0-acetyl-2,4(?)-di-0-methyl-β-D-glucopyranosyluronate)-2-acetamido-2-deoxy-4,6-di-0-methyl-α-D-glucopyranoside (I). The purity of I was ascertained by saponification, followed by esterification with diazomethane, to give the crystalline methyl 3-0-(methyl 2,4(?)-di-0-methyl-β-D-glucopyranosyluronate)-2-acetamido-2-deoxy-4, 6-di-0-methyl-α-D-glucopyranoside (II), which was characterized by a crystalline amide; the same amide was obtained directly from I. Acetylation of II gave I, whereas the methanolysis of methylated methyl hyaluronate not followed by acetylation gave directly II. The partial structure of II was established by methylation to produce the crystalline methyl 3-0-(methyl 2, 3, 4-tri-0-methyl-β-D-glucopyranosyluronate)-2-acetamido-2-deoxy-4, 6-di-0-methyl-α-D-glucopyranoside. Its hydrolysis gave a dimethylglucosamine, identified by paper chromatography, demonstrating the linkage between the sugars to be of the glucuronidic type. Hydrolysis of I gave a sirupy dimethylglucosamine hydrochloride characterized by its transformation in a 65 % yield into a crystalline 2-hydroxynaphtylidene derivative, identical to the synthetic 2 deoxy-2-(2′-hydroxynaphtylidenamino)-4, 6-di-0-methyl-D-glucose. The sirupy dimethylglucosamine was also transformed in a 70 % yield into the crystalline methyl 2-acetamido-2-deoxy-4,6-di-0-methyl-α-D-glucopyranoside (III) (59, 60, 61). The yield in crystalline product III calculated from the native hyaluronic acid is about 30 % of the theoretical yield. Such results confirm the glucuronidic linkage 1-3 postulated after the periodate oxidation (52). In addition, it establishes the pyranose form of the glucosamine moiety. The isolation of a crystalline methylated disaccharide in so high a yield from a partially methylated polysaccharide shows definitely that the major part of hyaluronic acid is composed of a repeating unit made of glucuronic acid and glucosamine linked in positions 1-3, with the sugars alternating regularly. It shows also the great resistance of the glucuronidic linkage toward acidic hydrolysis. All those deductions are in agreement with the results obtained by K. Meyer *et al.* (see below) in the study of the degradation of native hyaluronic acid. The study of the glucuronic part of II, after reduction with sodium borohydride, is under way.

The degradation of hyaluronic acid by acidic and enzymatic means has been reported simultaneously by Isikawa (62) and by Rapport *et al.* (63). From pure hyaluronic acid isolated from umbilical cords, after degradation with oxalic acid, the former author obtained a disaccharide in a very low yield (1-2 %), isolated as the crystalline methyl ester of the heptaacetyl derivative. The same crystalline derivative was also obtained from vitreous hyaluronic acid in a better yield (64), and a crystalline cinchonidine salt, and the crystalline N-acetyl methylester derivative were described (65). The disaccharide was shown to be a β-glucuronide, because it was split by the action of a β-glucuronidase devoid of α-glucuronidase activity (65). Periodate oxidation of the disaccharide (55) did not afford definite clues as to the structure and no further chemical work came to the reviewer's attention.

The isolation of a crystalline disaccharide named hyalobiuronic acid, obtained by the action of testicular hyaluronidase, followed by acid hydrolysis, or by direct acid hydrolysis of umbilical cord hyaluronic acid, has been reported from Dr. K. Meyer's laboratory (63, 67): the yield was 61 % by the former procedure and about 30 % by the latter procedure. Hyalobiuronic acid was shown by Weissmann and K. Meyer (68) to possess the structure of a 3-0-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-glucose by the following sequence of reactions: esterification, followed by acetylation gave the methylester heptaacetyl derivative with the same m. p. and probably identical to the one described by Isikawa (62). This compound was obtained in a 40 % yield from hyaluronic acid. From hyalobiuronic acid, an amorphous methyl ester hydrochloride was prepared, which was in turn oxidized with mercuric oxide to give the partially crystalline glucuronosidoglucosaminic acid. It was reduced with sodium borohydride to a glucosidoglucosaminic acid in an overall yield of 20 % from hyalobiuronic acid. The glucosidoglucosaminic acid was

degraded to a glucosidoarabinose by ninhydrin and isolated as the crystalline 2-0-β-D-glucopyranosyl-D-arabinose heptaacetate, in a 30 % yield. The structure of this compound was ascertained through its synthesis from laminaribiose. The total yield from native hyaluronic acid was 4 % using the biological degradation or half that amount by purely chemical means.

Such work does not only corroborate previously described evidence for a glucuronidic linkage in position 1-3, but it establishes it firmly to be in the β-form. The resistance of the glucuronidic linkage to any hydrolytic scission is noteworthy, as it has also been encountered in chondroitin sulfuric acid and will probably be found in other uronic acid containing mucopolysaccharides.

Judging from the above, nothing seems to contradict a straight chain for hyaluronic acid. However, much is still to be done to complete the chemical picture of hyaluronic acid.

The problem of the state of hyaluronic acid and its possible link to protein has received much attention in these last years, but it is outside the scope of this report. Recent reviews (69) have described the progress up to date.

The action of hyaluronidases on hyaluronic acid has been recently reviewed (70). Their action, combined with the action of acid or other enzymes leads to the isolation of interesting fragments of hyaluronic acid (63, 71).

### *Chondroitin sulfuric acid*

*Isolation and physical properties.* — A crystalline salt of chondroitin sulfuric acid isolated from cartilage has been reported by Einbinder and Schubert. The methods described do not require a strong alkaline medium, give an undegraded product with an excellent yield, and can easily be applied to large scale preparations (72, 73).

A highly purified mucopolysaccharide was obtained by Mathews and Dorfman (74) by precipitation as a complex salt of cobalt. Molecular weight determination by osmotic pressure measurements of such material gave much lower values (*ca.* 40 000) as those previously reported.

The chondroitin sulfuric acid isolated from skin (K. Meyer's chondroitin sulfate B) seems to have a chemical constitution definitely different from the one isolated from cartilage, as shown by its high negative rotation (—60° C.), its different reaction with carbazol and its lability toward acid hydrolysis (12). An isomer of chondroitin sulfuric acid has been isolated from bovine nuclei pulposi and shown, by infra-red spectroscopy, to possess a different chemical structure from the one isolated from cartilage (48). A mucopolysaccharide having the same components as chondroitin sulfuric acid, but with a high negative rotation (—60° C.) and a definite anticoagulant activity, has been isolated from the mother liquors of the preparation of heparin and designated β-heparin (6).

*Chemical structure.* — Periodate oxidation of chondroitin sulfuric acid has been reported by many laboratories. Various results have been obtained, less than 0.2 moles (75, 76), 0.5 moles (77), and even 1 mole of oxidant used for each disaccharide unit (73, 78, 79). In the reviewer's laboratory, periodate oxidation led to no conclusive results. Amounts of periodate used varied from 0 to more than 2 moles per period, depending on the temperature, pH and excess of oxidant (80). As it has been demonstrated (28) that the 5-C of the glucuronic acid moiety is susceptible to oxidation, no conclusions concerning the structure can be reached by determination of its absence after oxidation.

Chondroitin sulfuric acid has been methylated in the cold with dimethyl sulfate and sodium hydroxide (75, 80). No chemical degradation seems to take place, as the analytical values for nitrogen, acetyl, sulfur and carboxyl are correct (80). After hydrolysis, studies of the methylated fragments were done by periodate oxidation, assuming that the mixture was composed of methylated monosaccharides (75). However, it has been shown in methylated hyaluronic acid that the glucuronosyl linkage was highly resistant to methanolysis (59), and it is also known that the same linkage in chondroitin sulfuric acid is resistant enough to give chondrosine (see below). It can be assumed that the above described mixture of methylated sugars was composed mainly of disaccharides and no valid conclusion could be obtained from its periodate oxidation.

Methylated chondroitin sulfuric acid, studied by Bray *et al.* (81), was too degraded to give useful information on the chemical structure of the high polymer.

In 1914 Hebting (82) isolated from an acid hydrolysate of chondroitin sulfuric acid a disaccharide as the crystalline ethyl ester hydrochloride; it is quite surprising that its chemical structure, which would have given major information on the structure of this mucopolysaccharide has not been subjected to a thorough investigation until recently. Twenty seven years later, Levene (83) reported the isolation of the crystalline methyl ester hydrochloride. It has been shown (84) to derive from the same disaccharide as the one described by Hebting. Levene prepared some crystalline derivatives and assigned to the disaccharide the structure of a galactosaminidoglucuronic acid, without strong experimental evidence (83). Wolfrom *et al.* (78) repeated the reduction, according to Levene, of the disaccharide to the corresponding glycitol, isolated as the crystalline methyl ester octaacetate, and also prepared the crystalline methyl ester heptabenzoate. The crystalline amide of the glycitol was oxidized with periodate and a structure was proposed, based on Levene's assumption (since then proven erroneous) that chondrosine was a galactosamidinoglucuronic acid.

In 1942, Masamune *et al.* (85) attributed to chondrosine the structure of a glucuronidogalactosamine compound, on the basis of an iodine oxidation similar to the one of galactosamine hydrochloride, and very different from the one of glucuronic acid. Additional evidence was obtained by a positive Morgan and Elson reaction of the crystalline N-acetyl derivative. After degradative oxidation to a non-nitrogenous product, glucuronic acid could be isolated and characterized. On the basis of the isolation of an uncharacterized formylglucuronic acid, the structure of a glucuronido-6-galactosamine was suggested (86). The disaccharide was shown to be a

β-anomer, by positive splitting with a β-glucuronidase devoid of α-glucuronidase activity (87). Periodate oxidation of the N-acetyl ethyl ester of chondrosine used 4 moles of oxidant and liberated one mole of formaldehyde. This led Masamune *et al.* (76) to attribute to chondrosine the formula of a 3-0-(β-D-glucopyranosyluronic acid)-2-deoxy-2-amino-D-galactose, on the grounds that N-acetylgalactosamine used 3 moles of oxidant. However, it has been observed in the reviewer's laboratory that N-acetylglucosamine uses 5 moles of oxidant (27), which would favor a glucuronidic linkage 1-4 for chondrosine. It is evident that a measure of the amount of periodate used by such a molecule, where the glucuronic acid part reacts also, does not provide a firm basis for establishing the chemical structure of the disaccharide.

Another proof of a glucuronidogalactosamine structure was obtained by preparation of a methylglycoside from the N-acetyl methyl ester derivative of chondrosine (61), whereas under the same conditions it was shown that chondrosine methyl ester does not react with methanolic hydrochloric acid (78). It is a long established fact that aminosugar hydrochlorides cannot be glycosidified, whereas the N-acetyl derivatives react easily. The glycoside was then fully methylated, but direct hydrolysis did not lead to a crystalline methylated galactosamine (84).

Davidson and K. Meyer (88) greatly improved the preparation of chondrosine and obtained it in crystalline form by using a column fractionation on an acidic resin. The crystalline methyl ester was reduced with sodium borohydride to give a glucosidogalactosaminitol, which could be split by β-glucosidase, confirming the β-anomeric form of the glycosidic bond. In order to provide further proof for the partial structure of the disaccharide, glucose was isolated as its crystalline pentacacetate in a 55 % yield. Deamination with ninhydrin of the crystalline methyl ester hydrochloride, followed by re-esterification and reduction with sodium borohydride gave 2-0-(β-D-glucopyranosyl)-D-lyxitol (89). Periodate oxidation of this compound used 4 moles of oxidant with the production of 2 moles of formic acid and one mole of formaldehyde. Such results are not only in agreement with the structure proposed by Masamune *et al.* (76), but they are also much more conclusive, being based not only on the amount of oxidant used, but also on the amount of the substances released.

The evidence for the formation of complexes of chondroitin sulfuric acid and proteins has been reviewed by K. Meyer (69) and the isolation of a stable complex has been reported by Shatton and Schubert (90).

### *Heparin*

*Isolation and physical properties.* — Mild extraction of heparin with salt solutions was reported by Snellman *et al.* (91). A determination of the molecular weight of a commercial preparation gave a value of approximately 16 000 (92).

Jorpes and Gardell (93) were able to isolate from the mother liquor of the preparation of heparin (trisulfate) a heparin-like mucopolysaccharide containing only one group of sulfate for each disaccharide unit. They believed that heparins are mucopolysaccharides possessing the same disaccharide unit, but with various amounts of sulfate groups in the same molecule. Thus the product isolated will give an average value of the amount of sulfate. If this is the case, attempts to isolate homogeneous products to study the chemical structure would be fruitless and progress would be made only when an undegraded desulfated heparin can be prepared.

*Chemical structure.* — After the pioneer work done by the groups of Charles, of K. Meyer, of Jorpes and of Wolfrom, on the determination of the components of heparin, the most important finding has been the linkage of one of the sulfate group with the amino group of the glucosamine moiety. In 1940, Masamune *et al.* (94) could not detect any acetyl group after acid hydrolysis; this fact was later confirmed by Wolfrom *et al.* (95). Six years later, Jorpes *et al.* (96) were able to show that the free amino group appeared during acid hydrolysis at the same rate as the free sulfuric acid and, therefore, suggested that the sulfate group was bound to the glucosamine moiety through a sulfamic linkage. Independently, K. H. Meyer and Schwartz reached the same conclusion, after a study of the acid hydrolysis of the sulfate groups in heparin, in glucosamine-N-sulfate and in glucose-6-sulfate. In addition, they obtained a nitroso derivative of heparin by treatment with nitrous acid, a typical reaction for sulfamate compounds (21).

Because of the large number of non-reactive groups, periodate oxidation of the native heparin does not provide clues as to its structure. Oxidation of a desulfated heparin was reported by Wolfrom *et al.* (97). Unfortunately, the degree of degradation was not reported and, in the opinion of the reviewer, oxidation of the extremities of the chains can easily lead to misinterpretation. Partial acid hydrolysis produced an amorphous reducing disaccharide containing one sulfate ester group, from which an amorphous N-acetyl derivative could be prepared. Assuming those products to be homogeneous and the disaccharide to be of the glucosaminidoglucuronic acid type, periodate oxidation was carried out on both derivatives. Based on the periodate oxidation of the undegraded polymer and of its products of degradation, a chemical structure was proposed for heparin.

Masamune *et al.* (98) also hydrolyzed heparin and obtained an amorphous fraction, which they assumed to be a homogeneous disaccharide, but no crystalline derivatives could be prepared. Its rotation was identical to the one reported by Wolfrom *et al.* (97), but no sulfur could be detected. On the basis of a positive Elson and Morgan reaction, the disaccharide was assumed to be of the glucuronidoglucosamine type.

No further investigations clarifying these discrepancies have been reported up to now. Complexes of heparin and proteins have been discussed by Gorter and Nanninga (99).

This last discussion of our lack of knowledge of the chemical structure of one of the biologically most important mucopolysaccharides shows the great number of problems which must still be solved to obtain some clear picture of the complete class of compounds. While

improved methods of isolation will increase the number of known mucopolysaccharides, even the general structure of the simplest member of the family has not been elucidated as yet. The possible relationship of mucopolysaccharides to pathological conditions of the connective tissue, the increasing interest in their biological action, and the recent discoveries in the metabolism of their two major components by the groups of Leloir, Kalckar and Strominger, will certainly be a great incentive to biochemists and organic chemists to provide a better understanding of the chemical structures of this interesting group of substances.

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## On the mode of combination of carbohydrate with amino acid grouping in mucopolysaccharides and mucoproteins

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(*Received* 26 *July* 1955)

As early as 1943 (1), I put forward before the Japanese Biochemical Society that the natural mucoproteins or so-called glycoproteins can be classified into 'glucidamins' as simple proteins and glycoproteins as real conjugated proteins. Glucidamins are proteins containing a hexosamine or hexosamines and a Molisch positive monosaccharide or monosaccharides but no hexuronic acid as sugar components. Most of them contain sialic acid judging from recent findings. Glycoproteins of my terminology possess in their molecule a hexuronic acid-containing polysaccharide with or without a polysaccharide or polysaccharides giving positive Molisch reaction. The ground on which the view was based will be accounted for. Chondromucoid, prepared according to Mörner (2) from whale nasal cartilage, is made up of chondroitin sulfuric acid and a glucidamin, whose carbohydrate moiety is constructed of acetylglucosamine, galactose and sulfuric acid in molecular ratios of 2 : 2 : 1 (3). On the other hand, umbilical mucin is a complex of a globulin-like protein (4) possessing no carbohydrate in the molecule and mucoitin (5), *i.e.* Meyer's hyaluronic acid. These mucoids were stirred up in water of various pH values, and the insoluble residues were found, as shown in figure 1, to differ as to chondroitin sulfuric acid or mucoitin content according to the acidity of the water, exclusive of the pH range below 2.0 with respect to chondroitin sulfuric acid. Detailed explanation will be left to the original article (6). Moreover, all the chondroitin sulfuric acid was removed from the glucidamin at pH 7.0 by treatment of the chondromucoid according to Sevag, and also the protein free from mucoitin was carried down by the aid of formaldehyde from the solution of funis mucin adjusted to pH 7.0 with baryum hydroxide (6). Limacoitin sulfuric acid (7) and heparin (8) could be also isolated from the respective glycoproteins in a similar manner. These data led us to conclude that the polysaccharides having a hexuronic acid and a hexosamine as sugar components are bound

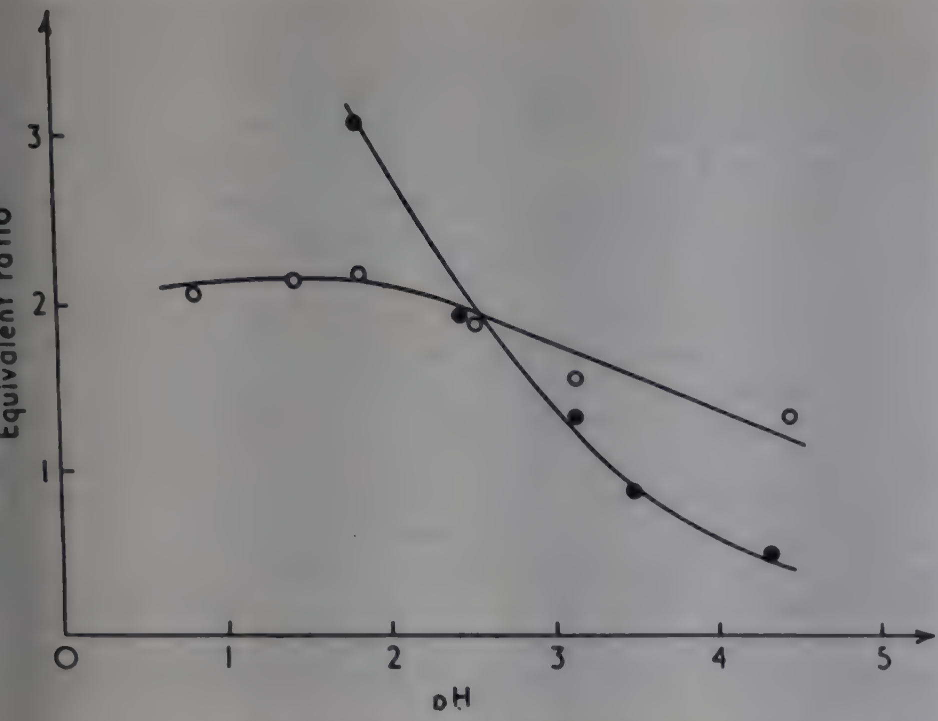


Fig. 1. — Equivalent ratios of associated mucoitin (●) and chondroitinsulfuric acid (○) as acids to total free basic protein nitrogen at different pH values (Masamune and Yasuoka).

with the protein by ionic attraction and additional co-ordination. Our experimental scheme may have been crude in the light of present advanced knowledge of chemistry, nevertheless, the view, I think, has been supported by or at least does not conflict with recent observations of Blix (9), of Partridge (10), of Ogsten and Stanier (11), of Gorter and Nanninga (12) and of Woodin (13), made by electrophoresis, viscosimetry, sedimentation and else, because I admitted the occurrence of co-ordinate linkage besides salt linkage. Meyer (14) suggested the chondroitin sulfuric acid-protein complex in hyaline cartilage to be of salt nature, but insisted that the itinsulfuric acids in skin, tendon and the like are « stably linked to protein », because they can not be isolated by gentle means. We revealed that the equivalent ratio of free basic nitrogen of hexone bases to acidic groups of chondroitin sulfuric acid is 0.9 in bull tracheal cartilage and only 0.3 in whale nasal cartilage. Hence, in these tissues, chondroitin sulfuric acid must be loosely bound and present in part as metal salt, but in bull tendon, the corresponding ratio reached even 11.2 so that the chondroitin sulfuric acid is pretty tightly fastened by proteins (15). Moreover, chondroitin sulfuric acid could be isolated from tendomucoid β (Sasaki) in pure state by the formalin method of Masamune and Osaki (16) without difficulty (17). The skin itinsulfuric acids are extracted with 10 % $CaCl_2$ readily from the tissue, if the latter is freed from collagen preliminarily by virtue of 50 % urea (18). After all, no fact seems to be contradictory to my conception.

On the contrary, the polysaccharides in glucidamins, namely, the Molisch-positive polysaccharides can not be freed from amino acids not only by the gentle means applied above but also by such pretty drastic treatments

Table I

*Group mucopolysaccharide MP-I and its derivatives*

| | N | Hexosamine as glucosamine (Blix) (total hexosamine) | Galactose | L-Fucose | Sialic acid (Werner and Odin) | Aspartic acid | Glutamic acid | Indirect-Osaki-Turumi pos. as N-acetylglucosamine | Iodine used as glucose | Minimum molecular weight | Ash |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | in % | | | | | | |
| Group mucopolysaccharide (MP-I) | 4.3 | 36.1 (38.6) | 26.6 | 11.5 | 7.3 | 2.8 | 4.5 | 8.6 | 5.1 | 3500 (*) | 0.8 |
| After treatment : | | | | | | | | | | | |
| of MP-I w. hot N $CH_3COOH$ for 1.5 h. (MP-I*a*) | 4.0 | 35.9 | 29.2 | 7.3 | 0.3 | 0.8 | 4.9 | 15.2 | 7.5 | 3200 (**) | 0.5 |
| of MP-I w. alkaline iodine (MP-I′) | 4.3 | 31.3 | 27.0 | 11.9 | 7.1 | | | 4.8 | 0 | 3450 (**) | 3.2 |
| of MP-I*a* w. alkaline iodine (MP-I*a*′) | 4.1 | 31.2 | 29.0 | 7.0 | | | | 1.4 | 0 | 3200 (**) | 4.6 |
| of MP-I w. $HgCl_2$ (MP-I*b*) | 3.9 | 36.1 | 32.1 | 13.0 | 0.2 | | | 7.4 | 7.8 | 2900 (**) | 2.2 |
| | | | | | In equivalents/equiv. wt. | | | | | | |
| MP-I | | 6.9 (7.6) | 5.2 | 2.4 | 0.8 | 0.7 | 1.1 | 1.4 | | | |
| MP-I*a* | | 6.3 | 5.2 | 1.4 | 0.03 | 0.3 | 1.1 | 2.2 | | | |
| MP-I′ | | 5.9 | 5.2 | 2.4 | 0.7 | | | 0.7 | | | |
| MP-I*a*′ | | 5.6 | 5.2 | 1.3 | | | | 0.2 | | | |
| MP-I*b* | | 5.9 | 5.2 | 2.2 | 0.02 | | | 1.0 | | | |

(*) Calculated from iodine use.
(**) Calculated under the assumption that 5.2 molecules of galactose are contained per unit molecule.

as with 2 N NaOH of room temperature, even if they are acidic due to the presence of the sulfuate residue as in those in the glucidamins of chondro- and osseomucoid (19). I attributed the conjugation in this kind of mucoprotein vaguely to normal co-valencies, when I delivered the mentioned lecture in 1943. The latest study of ours (20), however, pointed to participation of N-glycosidic and ether bonds in binding together the peptide and the carbohydrate chains in a group mucopolysaccharide from pig stomach mucus. I enter into somewhat circumstantial description in this respect.

The group substance was prepared by alkaline cleavage of pig stomach mucus as informed by Yosizawa (21). It will be referred to as MP-I. It was electrophoretically homogeneous and analyzed as shown in table I. A red color with a spectrum characteristic of acetylhexosamine developed on treatment of it with Ehrlich reagent after heating with 0.33 N sodium carbonate. We are used to call the test 'indirect Osaki-Turumi'. The intensity of the color corresponded to 1.4 molecules of acetylglucosamine per unit molecule of MP-I. After treatment with iodine, the substance gave the red colour reaching only half in intensity. The acetylhexosamine molecules, which reduced iodine, as well as all of the sialic acid were cleft off on standing the substance together with $HgCl_2$ in a solution, suggesting acetal links. The remaining indirect-Osaki-Turumi positive acetylhexosamine molecules must be bound at their 1-C. Ester linkage is excluded, because N NaOH was employed to prepare the group substance. Therefore an N- or O-glycosidic link is conceivable. To settle the question, the material was heated at 100° C. with normal acetic acid for 1.5 h., and when cold, dialyzed. The undialysed product (named MP-I*a*) was found to contain acetylhexosamine, L-fucose and sialic acid each less by approximately 1 molecule per unit molecule than MP-I. It is to be remarked that MP-I contained only about 1 molecule of sialic acid per unit molecule. It was further treated with alkaline iodine

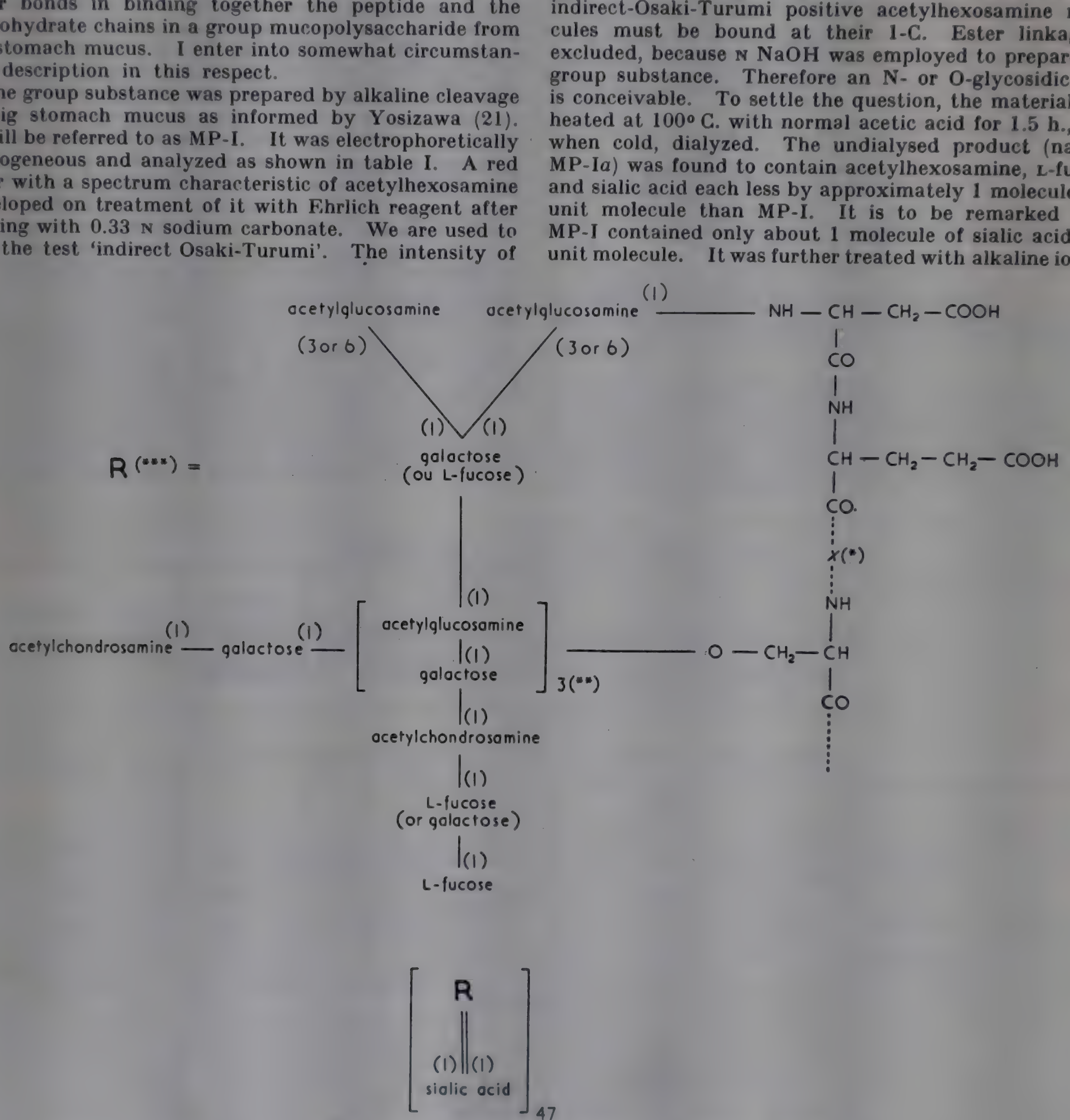


FIG. 2. — Group mucopolysaccharide MP-I (m. w. = 160 000-170 000).

(*) *X* signifies aminoacids varying from R to R;
(**) approximate number;
(***) the carbohydrate moiety is possible to change partially from one R to another.

TABLE II

*Separation of glucosamine-galactoside serine ether from the group muco-polysaccharide (MP-I)*

MP-I
- take up in water, boil with Dowex-1, filter
  - FILTRATE
    - boil with Dowex-50, filter
      - FILTRATE
        - dialyze, evaporate to dryness
        - MP-II
          - hydrolyze with N $H_2SO_4$ at 100° C., neutralize with $Ba(OH)_2$, centrifuge
            - SUPERNATANT
              - concentrate, pass through Dowex-50, wash the column with water
                - ELUATE + WASHINGS (non-amino sugars)
                - ADSORBED PART
                  - displace with 0.5 N $NH_4OH$, wash the column with dilute $(NH_4)_2CO_3$
                    - WASHING
                    - ELUATE
                      - bubble with $CO_2$
                      - distil to dryness
                      - RESIDUE
                        - extract with methanol
                          - SOLUTION
                            - distil to dryness
                            - RESIDUE
                              - dissolve in water-methanol, stir up with Dowex-1-carbonate, add acetic anhydride and stand, filter
                                - FILTRATE + WASHINGS
                                  - heat to boiling, cool, neutralize, pass through Dowex-50, wash the column with water
                                    - ELUATE + WASHINGS (acetylamino sugar, acetyl disaccharide)
                                    - ADSORBED PART
                                      - displace with 0.5 N ammonia
                                      - SOLUTION
                                        - distil *in vacuo*
                                        - RESIDUE
                                          - fraction w. chromatopack (Solv. butanol-acetic acid-water)
                                          - CRYSTALLINE PRODUCT

and named MP-Ia′. The product here was found to contain acetylhexosamine less by another 1 molecule. On the other hand, MP-I contained approximately 1 molecule each of glutamic and aspartic acid per unit molecule in addition to other amino acids. No amino acid-amino group of MP-I, exclusive of the one of guanidine nucleus, was reactive to dinitrofluorobenzene (DNFB). By the acetic acid treatment, a great part of the aspartic acid was lost, and the $NH_2$-group of glutamic acid residue reacted with the reagent DNFB. These and other findings suggested that, in each unit molecule of MP-I, one of the two terminal acetylhexosamine molecules is linked with $\alpha$-$NH_2$ group of aspartic acid standing at a terminal of the peptide chain. Further explanation of table I will be omitted. As will be illustrated below, the proper carbohydrate is also fixed to the peptide chain by an ether bond. Figure 2 is a provisional formula of the group mucopolysaccharide MP-I depicted from these data and others. Acetylglucosamine-galactose disaccharide residues in the brackets are acetylglucosamine-4-β-galactoside for the most part.

Next a successful attempt of demonstrating the ether bond will be given. As shown in table II, the group mucopolysaccharide was at first boiled with Dowex-1 and Dowex-50 in turn. Thereby the peptide chain was shortened, and only 2 amino acids, serine and alanine, remained in the undialysable residue (MP-II). This was heated with N $H_2SO_4$ at 100° C., and fractioned with Dowex-50 and methanol, and after acetylation in the presence of water, fractioned furtheron with Dowex-50 and a chromatopack. Finally, crystals in plates of acetylglucosamine-galactoside-serine-ether were obtained (figure 3). The product decomposed at 145° C. with gas

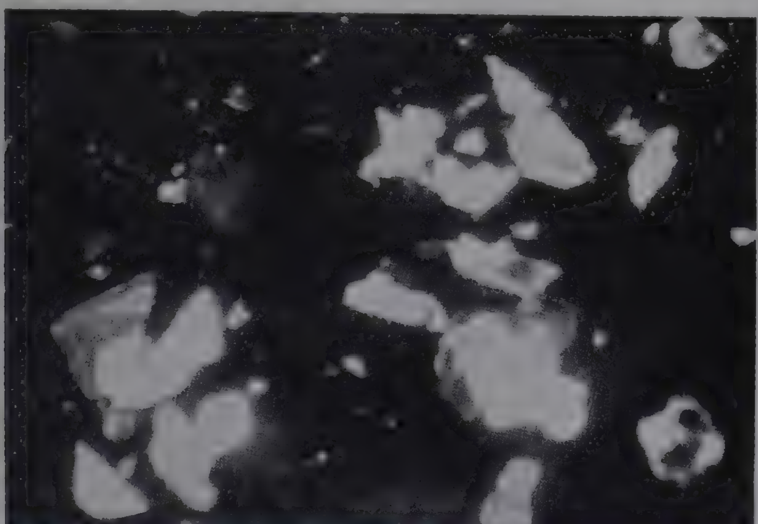

Fig. 3. — Microphotograph (polarized light) of crystals of acetylglucosamine-galactoside serine ether.

evolution. It had $[\alpha]_D^{20}$ in water of +53.2° (5 min. after dissolution) and gave the positive indirect-Osaki-Turumi and ninhydrin reaction. Analyses approximated theory (table III). Since serine was liberated, when the ether was boiled for 24 hours with 6 N HCl, it must be combined with the galactose residue.

Now I return to the story regarding classification of mucoproteins or socalled glycoproteins.

Nucleo- and chromoproteins are well-defined conjugated proteins, because the conjugation in their molecule

TABLE III

*N-Acetylglucosamine-galactoside serine ether*

| | Found | Calc. for $C_{17}H_{28}O_{13}N_2 . H_2O$ |
|---|---|---|
| Crystal water | 3.5 % | 3.64 % |
| N | 5.9 % | 5.7 % |
| Galactose | 36.6 % | 36.9 % |
| Glucosamine | 37.9 % | 36.6 % |
| Iodine use as glucose | 38.2 % | 36.9 % |
| ml. of 0.01 N NaOH used in formol titration of : | | |
| 5.3 mg. substance | 1.15 | 1.09 |
| 7.9 mg. substance | 1.65 | 1.62 |

is effected by electrostatic attraction and coordination but not by normal co-valencies. The mucoproteins, in make up of which polyuronic acids participate, well resemble these conjugated proteins in the same aspect, and therefore they are assumed as real conjugated proteins and might be given the name of 'glycoprotein', that is suited to this kind of complex protein. Apart from mucoproteins, many coagulable proteins of blood serum, milk and bird eggs, phosphoproteins and plant simple proteins, all of which belong to the group of simple proteins, have been shown to have a stably

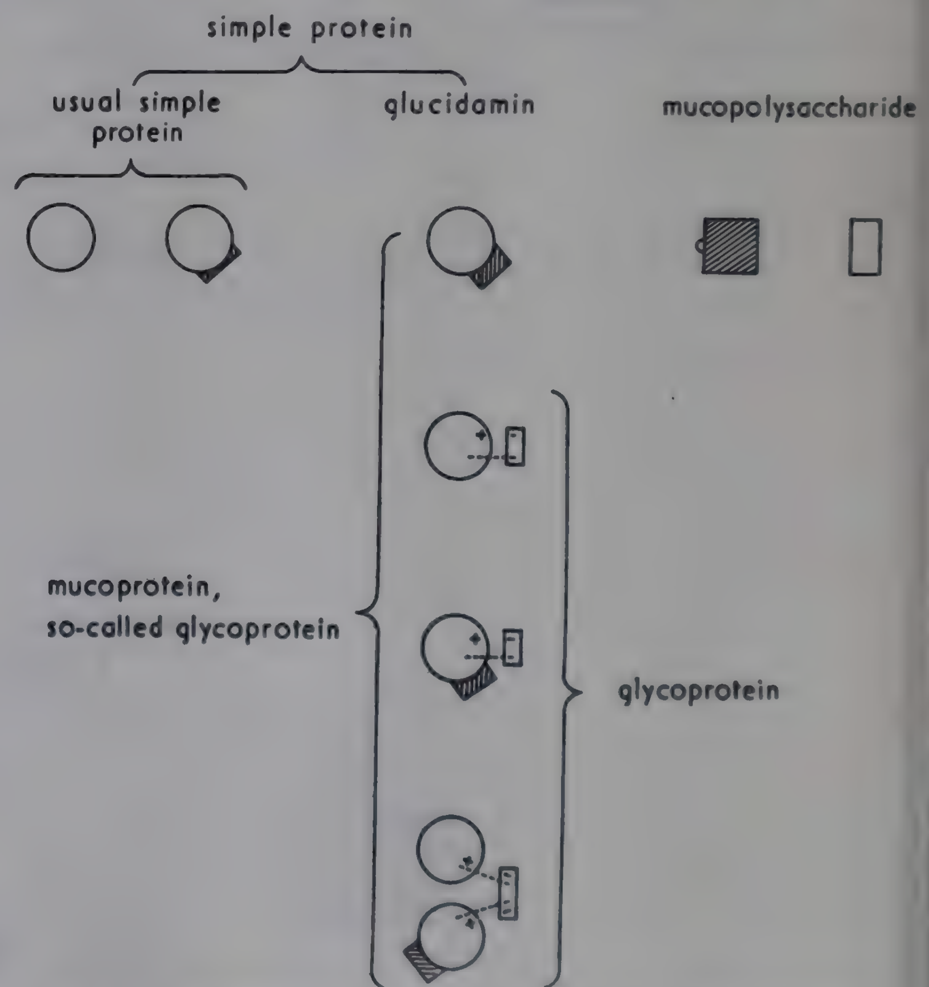


Fig. 4. — Classification of mucoproteins and mucopolysaccharides, respresented graphically. Open rectangles indicate polysaccharides containing hexosamine and hexuronic acid, and rectangles with oblique lines Molisch-positive hexosamine-containing polysaccharides Circles and half circles mean amino-acid groupings. Dotted lines show coordinate bonds.

ttached carbohydrate composed of a hexosamine or exosamines, a hexose or hexoses and sialic acid, though mall in amount. From these simple proteins, thus onsidered, not only such mucoproteins as ovomucoid nd the mucin from snail foot but also the ones, which ssociate with an itin or itinsulfuric acid or heparin into uch glycoproteins as chondromucoid, saliva mucin and thers, differ only quantitatively but not qualitatively. Not all simple proteins have a carbohydrate, but, because of being bound together by normal co-valencies of the wo moieties, the carbohydrate-rich proteins of this kind might be more legitimately classed among simple proteins, as phosphoproteins used to be by English authors. We named those containing 10 to 50 % carbohydrate 'glucidamins' (1, 22). Figure 4 is a graphical representation of my proposal. Real glycoproteins contain usually a glucidamin as the constituting simple protein. Exceptions are those obtainable from umbilical cords (5) and vitreous humor (23), whose simple protein moiety gives amino acids alone on hydrolysis, and sclera mucoid β (24), in which the simple protein possesses less than 10 % carbohydrate. Saliva mucin (25) and scleramucoid α (24) are complicated and made up of a glucidamin, a simple protein with a minute quantity of carbohydrate, and mucoitin a glucuronic acid-containing polysaccharide. To add, Molisch-positive mucopolysaccharides contain more than 50 % carbohydrate, but none of them is devoid of amino acids, I think.

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# Sur quelques protéolyses limitées d'intérêt biologique

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(*Reçu le* 2 *juin* 1955)

## INTRODUCTION

### *Les protéolyses*

Avant de donner dans le présent rapport quelques exemples de protéolyses limitées, il convient de trouver pour ce terme une définition précise. Si l'on s'en tient au sens étymologique, une protéolyse est la dégradation d'une molécule protéique par rupture de certaines liaisons. Ce sens est donc très large puisqu'il ne précise ni la nature des liaisons rompues, ni l'agent causal de la dégradation, ni son mécanisme chimique.

Notre conception actuelle de la structure fondamentale des protéines repose sur l'existence de chaînes dans lesquelles des résidus d'aminoacides sont placés côte à côte et reliés par des liaisons peptidiques. Ce type de liaisons est certainement prédominant. Mais il n'est pas le seul. C'est donc par une convention somme toute arbitraire que l'on restreint généralement le domaine de la protéolyse à la rupture de liaisons peptidiques.

Il est bien connu en outre que les molécules protéiques sont dégradées sous les influences les plus diverses. Une deuxième convention réserve cependant le terme de protéolyse aux hydrolyses provoquées par des enzymes. En adoptant ces deux conventions, nous arrivons à la définition classique de la protéolyse : l'hydrolyse dans une protéine de liaisons peptidiques par un enzyme. L'étroitesse de cette définition résulte d'ailleurs beaucoup plus de la première convention que de la deuxième. Linderstrom-Lang (1) a maintes fois attiré l'attention sur l'existence probable d'une phase préparatoire dans l'attaque de certaines protéines (β-lactoglobuline) par la trypsine. Or, d'après l'auteur danois, cette phase aurait davantage les caractères de la dénaturation que ceux d'une protéolyse proprement dite. De plus, Perlmann (2, 3) a montré que certaines phosphatases sont capables d'arracher les radicaux orthophosphoriques de l'ovalbumine en la transformant en d'autres protéines électrophorétiquement distinctes. Il s'agit bien là de l'hydrolyse d'une protéine par un enzyme. Mais nous sommes loin d'une dégradation protéolytique au sens conventionnel du terme.

### *Les protéolyses limitées*

Les travaux déjà cités de Perlmann (2, 3) indiquent clairement que l'ovalbumine peut subir une dégradation enzymatique limitée. Le caractère limité de la dégradation n'est d'ailleurs pas étonnant puisque, dans ce cas, deux liaisons seulement de la protéine satisfont les besoins structuraux des enzymes. Quand ces deux liaisons sont coupées, la réaction s'arrête tout naturellement. Par contre, les liaisons peptidiques sont beaucoup plus nombreuses. On doit donc s'attendre à ce que les enzymes protéolytiques proprement dits provoquent une dégradation beaucoup plus profonde. On sait néanmoins que ces enzymes sont incapables de couper toutes les liaisons peptidiques des protéines. Avec les endopeptidases (4), on obtient en fait deux types de réactions : ou bien la réaction dite « un par un » (5) au cours de laquelle l'enzyme semble attaquer chaque molécule protéique aussi loin que possible avant de passer à la suivante. Le mélange est alors constitué de protéine encore intacte et de peptides relativement petits ; ou bien la réaction que Linderstrom-Lang (1) appelle de façon imagée « fermeture éclair », au cours de laquelle l'enzyme semble convertir très rapidement toutes les molécules protéiques en peptides relativement gros, puis attaquer à nouveau ces peptides pour les convertir peu à peu en peptides plus petits (7). Le mélange contient alors des gros et des petits peptides (*). D'après Linderstrom-Lang (8), l'appartenance de la réaction à l'un ou l'autre type serait déterminée par la vitesse relative de la phase préparatoire et de l'attaque endopeptidasique. Quand la première est lente, la réaction est « un par un ». Dans le cas contraire, la réaction est en « fermeture éclair ».

Nous venons de voir que la réaction « fermeture éclair » comprend deux étapes : une étape rapide qui convertit les protéines en gros peptides, et une étape plus lente qui convertit les gros peptides en petits peptides. Si l'on pousse maintenant les choses à l'extrême, si l'on considère que ces gros peptides sont tellement gros que leurs dimensions et leurs propriétés fondamentales sont analogues à celles de la protéine initiale, nous arrivons au domaine que nous désirons depuis le début définir : celui de la protéolyse limitée. Autrement dit, une protéolyse sera dite limitée quand, au terme de sa première étape, une ou plusieurs des substances engendrées méritent encore d'être appelées des protéines. Si la structure de la molé-

(*) Afin de ne pas alourdir l'exposé, nous laissons de côté le cas où l'enzyme paraît rencontrer dans la molécule protéique un noyau particulièrement résistant (attaque de l'insuline par la chymotrypsine par exemple, 6).

cule est simplement modifiée, nous aurons une réaction du type :

Protéine A → Protéine B

ou une suite de réactions consécutives :

Protéine A → Protéine B → Protéine C ...

Si, au contraire, la réaction prive la molécule de certains de ses éléments, nous aurons :

Protéine A → Protéine B + $p_1$ → Protéine C + $p_2$ ... etc.

Nous n'envisagerons ici que les protéolyses limitées qui sont, ou tout au moins qui semblent être, provoquées par la rupture de liaisons peptidiques. Ce genre de protéolyse nous paraît en effet revêtir une importance particulière, et ceci pour deux raisons principales :

— L'enzyme protéolytique manifeste une spécificité tout à fait remarquable en choisissant quelques liaisons parmi une centaine ou un millier d'autres identiques ou analogues. Ces liaisons sont rapidement coupées tandis que la protéine-mère donne naissance à une ou plusieurs protéines-filles. Si l'on attend assez longtemps ou si l'on modifie les conditions opératoires, ces dernières peuvent d'ailleurs être attaquées à leur tour et donner naissance à des peptides. Elles survivent néanmoins assez longtemps pour manifester éventuellement leurs propriétés caractéristiques et pour pouvoir être isolées avec un bon rendement.

— Les protéines-filles jouent quelquefois dans l'organisme un rôle bien défini. Leur formation et par conséquent le processus de protéolyse limitée qui les engendre répond alors à une nécessité physiologique précise.

Trois exemples de protéolyses limitées seront discutés : la conversion de l'ovalbumine en plakalbumine qui servira en quelque sorte de préambule, la conversion du fibrinogène soluble en fibrine insoluble et capable de former un réseau tridimensionnel, la conversion en endopeptidases actives des deux précurseurs inactifs du pancréas : le chymotrypsinogène et le trypsinogène (*).

## LA CONVERSION OVALBUMINE-PLAKALBUMINE

### *Caractères généraux de la conversion*

Par un hasard heureux, Linderstrom-Lang et Ottesen (10, 11) essayèrent en 1947 de cristalliser de l'ovalbumine à partir de solutions abandonnées à +1° C. dans des conditions non-stériles pendant plusieurs mois. Au lieu d'être des aiguilles ovales comme d'habitude, les cristaux affectèrent la forme de plaques rectangulaires minces. La substance qui formait ces plaques possédait encore toutes les propriétés classiques des protéines. Mais c'était une protéine différente de l'ovalbumine puisque sa solubilité dans le sulfate d'ammonium était bien supérieure (12). Les auteurs notèrent d'autre part que toutes les solutions cristallisant en plaques étaient infectées par *Bacillus subtilis* alors que les solutions stériles continuaient à donner des aiguilles. Il était donc clair qu'un enzyme de la bactérie avait transformé l'ovalbumine en une nouvelle protéine que l'on appela plakalbumine (10). Une préparation active de l'enzyme en question fut bientôt obtenue par précipitation alcoolique des milieux de culture de la bactérie.

Les courbes de la figure 1 montrent que cette transformation est bien définie. Les ordonnées de la figure correspondent au nombre de ml. de $(NH_4)_2SO_4$ saturé que l'on doit ajouter au mélange pour qu'un premier trouble apparaisse. Etant donné la forte solubilité de la plakalbumine, ce nombre est une expression du degré de la conversion. Il atteint une limite précise et indépendante de la quantité d'enzyme utilisée.

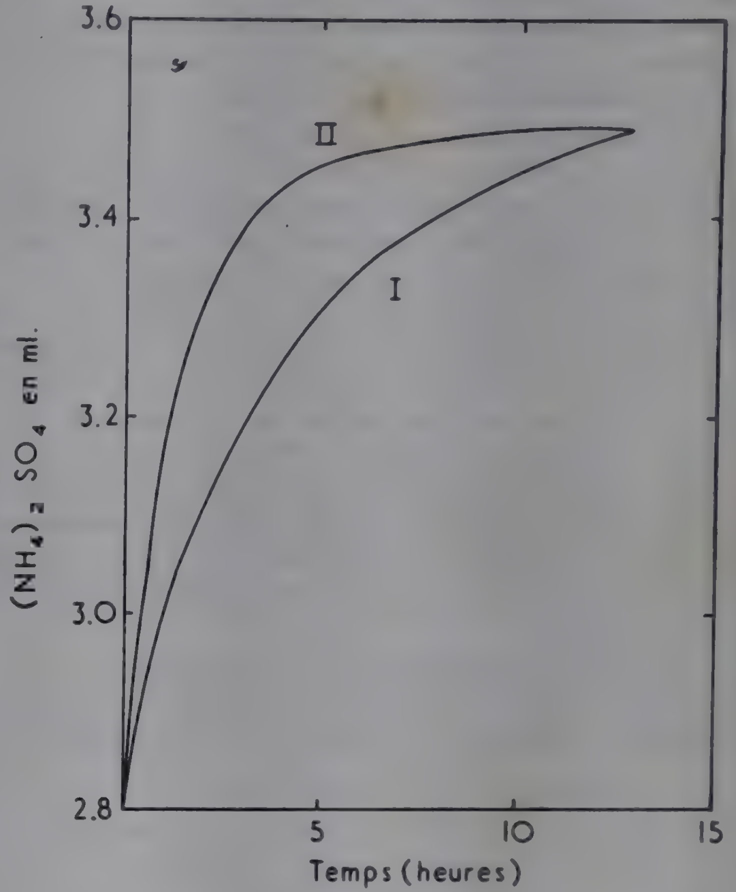



Fig. 1. — Cinétique de la conversion ovalbumine → plakalbumine, d'après Linderstrom-Lang et Ottesen (11)., Solutions à 5 % d'ovalbumine pH = 6.4. Température : 20° C. I et II : 8.8 et 17.6 µl. de solution enzymatique.

Deux faits montrent que la transformation est bien due à une dégradation de la molécule d'ovalbumine et que cette dégradation est limitée. D'une part, le poids moléculaire de la plakalbumine (44 400-44 600 par osmométrie (13); 43 700 par ultracentrifugation (11)) semble un peu inférieur (*) à celui de l'ovalbumine (44 900-45 100 par osmométrie (13); 44 000 par ultracentrifugation (11)). D'autre part, quelques petits peptides sont libérés pendant la transformation. La conversion ovalbumine-plakalbumine est donc, selon toute vraisemblance une protéolyse dans le sens large

(*) Malgré tout son intérêt, la discussion du mécanisme de l'activation du pepsinogène (9) nous semble encore prématurée. L'attaque des protéines par la carboxypeptidase est aussi un exemple très suggestif de protéolyse limitée. Nous ne la discuterons pas faute de place.

(*) La différence de quelques centaines d'unités pourrait paraître a priori peu significative, étant donné l'imprécision des techniques de mesure. Mais il faut reconnaître qu'elle correspond presque exactement au poids moléculaire de l'hexapeptide libéré pendant la conversion (voir plus loin).

du terme. Mais est-elle réellement due à la scission de liaisons peptidiques?

Si, tel est bien le cas, des groupements α-aminés et α-carboxyliques doivent apparaître et ils doivent en principe être décelables par les techniques classiques de la titrimétrie ou des résidus terminaux. Il faut d'ailleurs reconnaître que les techniques titrimétriques sont assez imprécises, tout au moins dans des mains inexpertes. Cette imprécision est particulièrement gênante dans le cas des protéolyses limitées où, par définition même, le nombre des ruptures est peu élevé. En outre, ces techniques fournissent des résultats globaux. Elles sont donc incapables de différencier les ruptures spécifiques (qui se produisent à côté de résidus bien déterminés) et les ruptures non-spécifiques (qui se produisent en principe au hasard). Linderstrom-Lang et Ottesen ne les ont donc pas utilisées (*). A priori, les techniques pour la détermination des résidus terminaux doivent être considérées comme beaucoup plus discriminatoires et efficaces. Leurs performances pourront d'ailleurs être jugées un peu plus loin. Mais dans le cas qui nous occupe ici, elles donnent des résultats surprenants, lesquels confèrent à l'ensemble du phénomène un caractère assez mystérieux.

Aucun résidu N-terminal n'a en effet été décelé jusqu'ici dans l'ovalbumine (15, 16) et la plakalbumine (11). Un résidu C-terminal d'alanine a bien été trouvé dans l'ovalbumine par des méthodes chimiques (17, 18). Mais, chose curieuse, la carboxypeptidase ne libère aucun aminoacide dans la plakalbumine (19, 20). Ces faits seront plus amplement discutés tout à l'heure. Nous devons dès maintenant retenir que les arguments directs en faveur du caractère protéolytique de la conversion font défaut.

Les arguments indirects sont au nombre de deux. En premier lieu, comme le montre la courbe de la figure 2, la conversion engendre de l'azote non-protéique et par conséquent des peptides. D'autre part, l'enzyme responsable de la conversion (la subtilisine) a été récemment cristallisé (21). C'est un enzyme protéolytique extrêmement actif qui coupe de nombreuses liaisons dans une série d'autres protéines. Ces deux arguments ne sont malheureusement pas tout à fait convaincants.

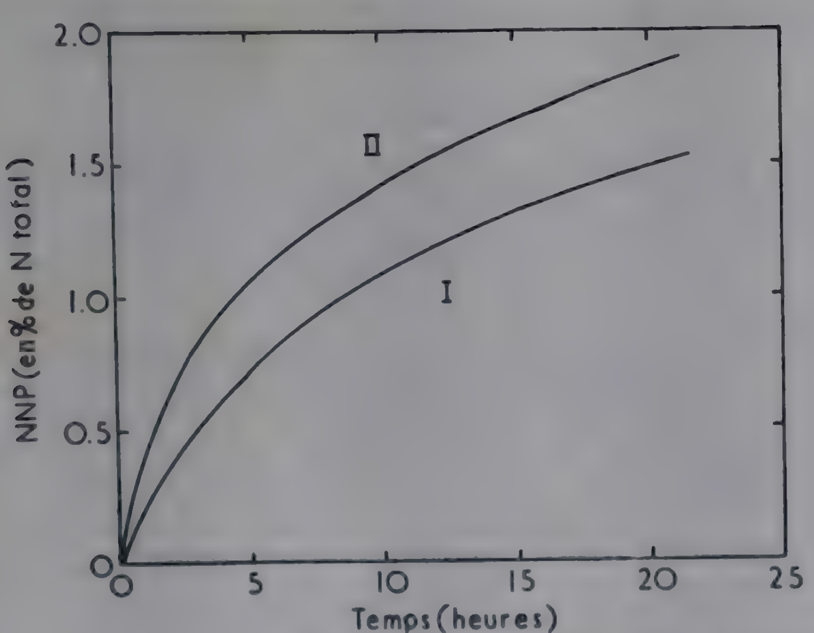



FIG. 2. — Formation d'azote non-protéique pendant la conversion, d'après Linderstrom-Lang et Ottesen (11). Pour les conditions expérimentales, voir légende de la figure 1. NNP = azote non protéique.

(*) Pourtant, à la même époque, Jacobsen (14) a obtenu certains renseignements intéressants en appliquant les techniques titrimétriques à l'étude de l'activation du chymotrypsinogène (voir plus loin).

### *Les peptides libérés pendant la conversion*

Dans la meilleure hypothèse, la protéolyse limitée représente l'essentiel du phénomène ; mais elle est toujours accompagnée de processus parasites dus à la dégradation non-spécifique de la protéine-mère ou à la dégradation ultérieure des protéines-filles. Quand le milieu réactionnel contient des peptides la première question est donc de savoir si ces peptides sont ou non spécifiques. La courbe de la figure 3 indique les relations existant au début de la réaction entre la quantité d'azote non-protéique formée et le rendement de la conversion. Elle est intéressante à plus d'un titre.

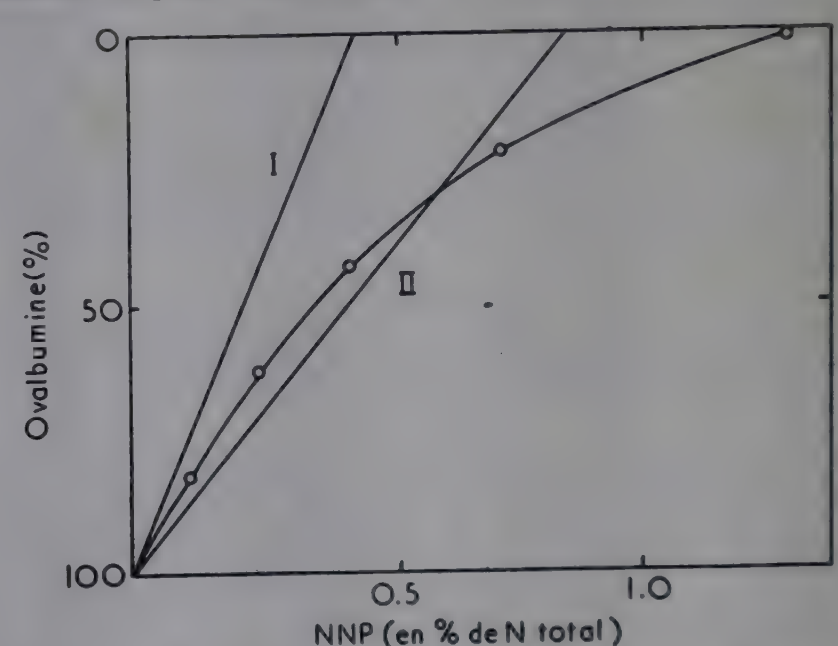



FIG. 3. — Proportion d'ovalbumine restante en fonction de l'azote non-protéique libéré, d'après Linderstrom-Lang et Ottesen (4).

Quels que soient le pH et la concentration de l'enzyme, les points représentatifs sont en effet situés sur une courbe unique. Nous avons donc affaire à une production massive de peptides spécifiques dont l'importance est telle, tout au moins au début de la réaction, qu'elle masque la production éventuelle de peptides non-spécifiques. La conversion ovalbumine-plakalbumine est en outre complète quand l'azote non-protéique atteint environ 1.2 % de l'azote total. Il faut donc prévoir que 6 atomes d'azote par mole sont libérés pendant la réaction spécifique. Enfin, la courbe est loin d'être rectiligne comme le voudrait l'équation simple :

$$\text{Ovalbumine} \rightarrow \text{Plakalbumine} + \text{peptide(s)}$$

Il est donc vraisemblable que la conversion s'effectue en plusieurs étapes consécutives. Si les étapes sont au nombre de deux, nous pouvons écrire (11, 12) :

$$\text{Ovalbumine} \rightarrow \text{Plakalbumine I} + \text{peptide(s) A}$$

$$\text{Plakalbumine I} \rightarrow \text{Plakalbumine II} + \text{peptide(s) B}$$

ou :

Ovalbumine → Plakalbumine I + peptide(s) A
Ovalbumine → Plakalbumine II + peptide(s) B

Plakalbumine I → Plakalbumine II + peptide(s) C

Nous allons voir que ce dernier schéma est en bon accord avec les résultats fournis par l'analyse des peptides.

Ottesen et Villee (22) ont chromatographié dans des colonnes d'amidon le mélange de peptides engendré par la conversion et ils ont obtenu une série de diagrammes dont les plus significatifs sont reproduits dans la figure 4. La technique de chromatographie dans l'amidon est d'ailleurs incapable de fractionner les peptides de façon bien efficace. Néanmoins, les mélanges étudiés semblent contenir trois fractions principales auxquelles correspondent au moins trois peptides. Deux d'entre eux (peptides A et B) apparaissent au début. Le troisième (peptide C) ne se forme qu'après un certain laps de temps.

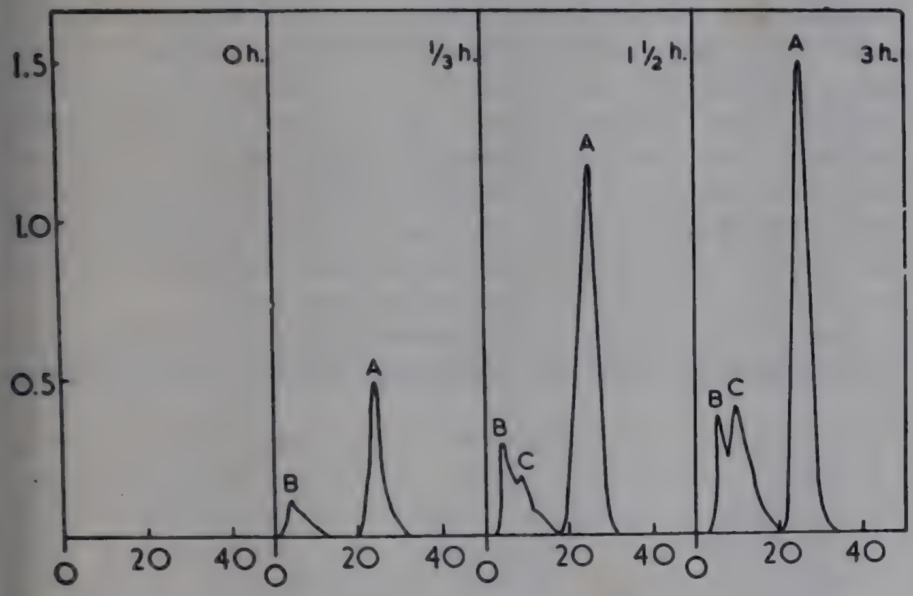
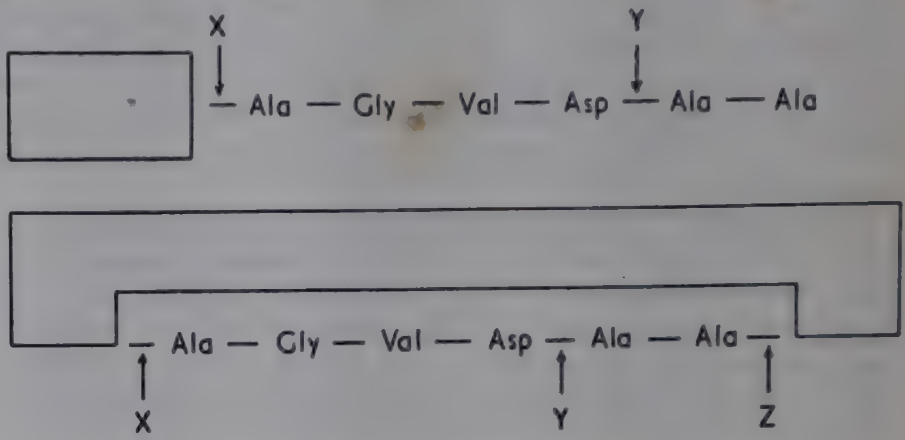



FIG. 4 — Chromatographie sur amidon des peptides engendrés par la conversion, d'après Ottesen et Villee (22). Ordonnées : coefficients d'extinction. Abscisses : n° des fractions (0.6 ml. environ).

Ottesen et Wollenberger (23) ont étudié la composition et la structure de ces trois peptides. Le peptide A fut bientôt identifié comme étant l'alanylalanine (Ala.Ala). La structure du peptide C fut trouvée sans difficulté particulière grâce à la technique de dégradation récurrente d'Edman (Ala. Gly.Val.Asp.). La formule brute du peptide B était ($Ala_2$.Gly.Val.Asp.), qui correspond à la somme des éléments des deux peptides précédents. Le problème se résumait donc à savoir si, pour construire cet hexapeptide, il convenait d'ajouter le dipeptide Ala.Ala au début ou à la fin du tétrapeptide. En appliquant la technique d'Edman à l'hexapeptide, on libère d'abord la thiohydantoïne de l'alanine, puis simultanément, la thiohydantoïne du glycocolle et le dipeptide (Ala.Ala). Il est donc très probable que l'enchaînement de l'hexapeptide soit (Ala.Gly.Val.Asp.Ala.Ala) et que la liaison Asp-Ala soit fortuitement rompue pendant la cyclisation de la deuxième thiohydantoïne.

### *Existence probable d'une phase préliminaire dans la conversion*

Les résultats qui viennent d'être examinés signifient vraisemblablement qu'il existe quelque part dans la molécule d'ovalbumine une séquence Ala.Gly.Val.Asp. Ala.Ala. Cette séquence peut être reliée au reste de la molécule par une de ses extrémités ou par les deux (1). Dans le premier cas, la plakalbumine I serait formée grâce à la rupture de la liaison mal définie existant entre le groupe amine du résidu d'alanine de gauche avec le reste de la molécule (point X). Dans le deuxième cas, il faudrait imaginer un processus supplémentaire et préliminaire au point Z, libérant ou démasquant le résidu d'alanine de droite.




En fait, la première hypothèse apparut vraisemblable quand Steinberg (24) annonça que la carboxypeptidase cristallisée libère des quantités substantielles d'alanine à partir de l'ovalbumine. Mais une incubation préalable de l'enzyme avec le di*iso*propylfluorophosphate (qui inhibe non pas la carboxypeptidase elle-même, mais les impuretés endopeptidasiques qu'elle peut contenir) supprime complètement cette propriété. Le résidu d'alanine de droite n'est donc pas C-terminal au sens ordinaire du terme ou il est inaccessible à la carboxypeptidase pour une raison quelconque. C'est donc la deuxième hypothèse qu'il faut envisager, avec l'obligation d'un processus préliminaire au point Z. La structure de la molécule à ce point Z n'est d'ailleurs pas mieux connue que celle au point X.

Dans deux publications ultérieures, Steinberg (20, 25) a montré que l'impureté endopeptidasique de la carboxypeptidase cristallisée est vraisemblablement identique, ou tout au moins très analogue, à la subtilisine. Il a du même coup découvert une troisième plakalbumine, la plakalbumine III. Nous venons de voir en effet que la carboxypeptidase cristallisée perd son aptitude à libérer de l'alanine quand on l'incube avec du di*iso*propylfluorophosphate. Mais, si l'on ajoute à l'enzyme traité une trace de subtilisine, l'alanine est à nouveau libérée et l'ovalbumine est transformée en une protéine cristallisant en plaques. Ce représentant supplémentaire de la famille des plakalbumines ne diffère apparemment de l'ovalbumine que par un résidu d'alanine en moins.

Ovalbumine → Plakalbumine III + alanine

Les résultats obtenus par Steinberg sont fort intéressants. On peut admettre, bien que nous ne disposions pas encore de preuves formelles à ce sujet, que le premier

effet de la subtilisine soit de modifier la structure de la molécule au point Z de façon telle que le résidu d'alanine soit libéré ou démasqué. Si les quantités de subtilisine sont très faibles, la protéine formée (qui est peut-être déjà une plakalbumine) est suffisamment stable pour perdre son alanine C-terminale au contact de la carboxypeptidase. Mais, si les quantités de subtilisine sont plus fortes, cette protéine est rapidement attaquée au point Y et au point X pour donner les plakalbumines I et II. Toutefois, les différences d'interprétation commencent quand on se demande quelle est la modification de structure que réalise la subtilisine au point Z. Est-ce la rupture d'une liaison peptidique Ala-X? La protéine primaire et toutes les plakalbumines devraient alors posséder un résidu N-terminal X que l'on n'a pas découvert jusqu'ici. Est-ce la rupture d'une autre liaison covalente ou secondaire? Dans ce dernier cas, on se trouverait en présence d'un exemple remarquablement suggestif de cette phase « préparatoire » à la protéolyse, dont l'existence est postulée par Linderstrom-Lang depuis de nombreuses années.

En somme, Linderstrom-Lang et ses collaborateurs ont étudié un phénomène d'une importance fondamentale qui, grâce justement à ses caractères surprenants, est peut-être susceptible de nous faire découvrir à brève échéance des aspects inconnus de la structure des protéines et de la dégradation de ces molécules par les enzymes.

## LA CONVERSION DU FIBRINOGÈNE EN FIBRINE

On sait que la coagulation du sang est liée à la transformation d'une protéine soluble du plasma, le fibrinogène, en une autre protéine insoluble et susceptible de former un gel à structure fibrillaire, la fibrine. Cette fibrine n'est d'ailleurs pas le terme ultime de la transformation. Elle semble subir d'abord quelques modifications de structure grâce aux ions calcium et à un facteur plasmatique stabilisant. Ces modifications lui font perdre sa solubilité dans l'urée 5 M (26). Elle est susceptible également d'être attaquée par divers enzymes protéolytiques. La formation de cette fibrine représente malgré tout une étape relativement bien définie dans une chaîne plus complexe de réactions.

Depuis de nombreuses années, tout le monde est d'accord pour penser que la fibrine doit sa formation à l'action de la thrombine sur le fibrinogène et que la structure du fibrinogène subit dans cette affaire un changement permanent. La fibrine coagule en effet, même en l'absence de thrombine, dès que le pH et la force ionique de sa solution prennent une valeur convenable. Le changement toutefois doit être minime. La viscosité de la fibrine en solution dans l'urée (27, 28), sa vitesse de sédimentation dans un champ centrifuge (29) et son pouvoir de diffusion sur la lumière (30) sont en effet analogues à ceux du fibrinogène. La composition en aminoacides des deux protéines est en outre très voisine (31). Mais, dès qu'on se préoccupe de préciser l'origine du changement, on retombe dans l'incertitude si bien illustrée par l'exemple de la plakalbumine : la dégradation du fibrinogène par la thrombine est-elle due à la rupture de liaisons peptidiques ou à la rupture d'autres liaisons? La première hypothèse a d'ailleurs été adoptée d'emblée par les anciens auteurs (32, 33, 34). Mais certains (35, 36) en ont plus récemment contesté la validité. Jusqu'à ces toutes dernières années, les arguments expérimentaux en sa faveur étaient d'ailleurs peu nombreux et fort indirects. On savait bien par exemple que d'authentiques protéases comme la papaïne (37) sont capables de coaguler le fibrinogène et que la thrombine paraît elle-même se comporter comme une protéase vis-à-vis de certaines protéines (fibrine, caséine, 38, 39) et vis-à-vis de la tosyl-arginine-amide (40) Par contre, on savait que la thrombine ne coupe aucune liaison peptidique dans l'ovalbumine ainsi que dans la myosine (41) et, comme la fibrine semble contenir moins de glucides que le fibrinogène (42) (voir par contre, 43), on pouvait penser que la dégradation affecte une région non-peptidique de la molécule.

Bailey et Bettelheim, Lorand et Middlebrook (41) ont contribué récemment à éclaircir un peu le problème. Ces auteurs ont tout d'abord signalé (41) que certaines différences de structure entre le fibrinogène et la fibrine peuvent être mises en évidence grâce à l'emploi de la technique de Sanger. Malgré des divergences assez sérieuses entre les résultats initialement présentés par les deux groupes, on peut tenir pour acquis (41, 44, 45) que le fibrinogène possède en position N-terminale de la tyrosine et de l'acide glutamique, alors que la tyrosine et le glycocolle occupent cette position dans la fibrine. Préciser avec une exactitude rigoureuse le nombre de ces divers résidus dans des molécules aussi grosses est d'ailleurs difficile. Nous retiendrons donc simplement que les deux protéines semblent posséder le même nombre de résidus de tyrosine et que les résidus d'acide glutamique du fibrinogène paraissent remplacés dans la fibrine par un nombre plus élevé de résidus de glycocolle.

Quoi qu'il en soit, le fait même que l'acide glutamique ait été remplacé par du glycocolle pendant la conversion

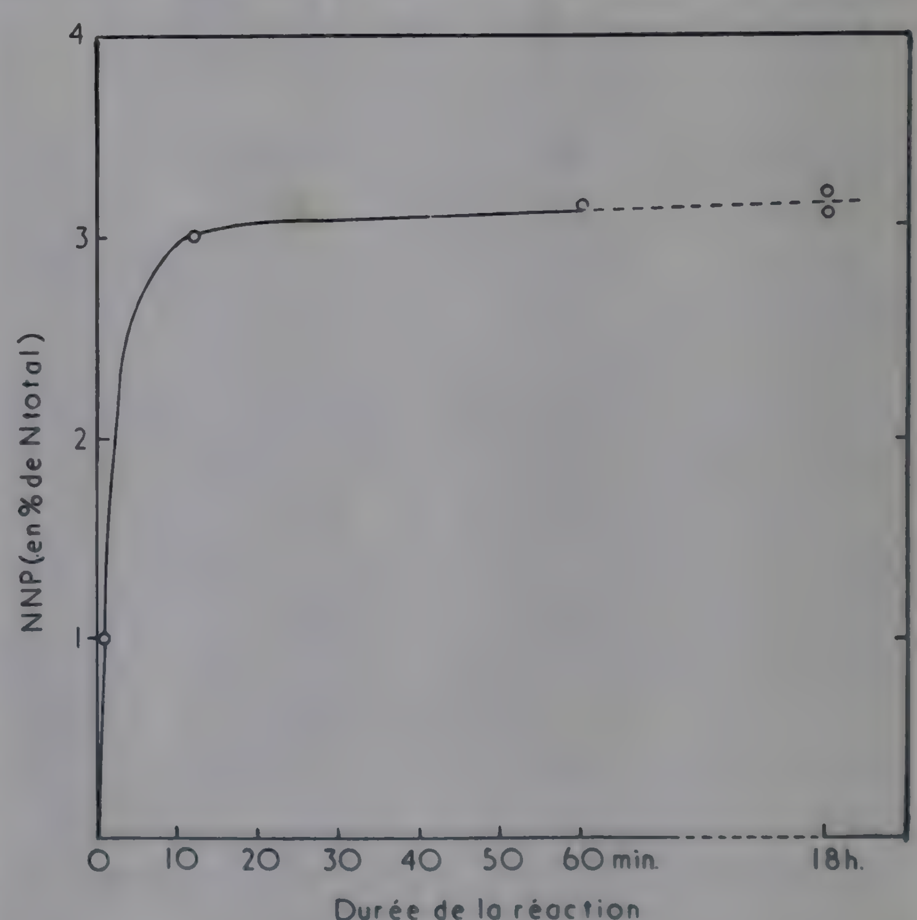



FIG. 5. — Formation d'azote non-protéique pendant la conversion du fibrinogène en fibrine, d'après Lorand (46).

suggère fortement que cette conversion résulte de la scission de liaisons peptidiques possédant du glycocolle sur leur flanc aminé et que des peptides contenant l'acide glutamique perdu sont libérés.

Comme le montre la figure 5, une quantité définie d'azote non-protéique, dont une partie tout au moins est attribuable à la conversion, est effectivement formée. En outre, cette fraction non-protéique possède tout l'acide glutamique N-terminal perdu par le fibrinogène (45). Elle contient donc le ou les peptides cherchés.

L'étude de cette fraction non-protéique n'est malheureusement pas encore très avancée. Lorand (46, 47) a proclamé à plusieurs reprises qu'elle est constituée par un seul peptide, le fibrino-peptide. Mais aucun argument expérimental ne vient le démontrer. Bien au contraire, l'électrophorèse sur papier permet de séparer au moins deux peptides de composition tout à fait différente (45). L'un renferme l'acide glutamique N-terminal du fibrinogène. L'autre paraît dépourvu de résidu N-terminal. Il contient par contre toute la lysine de la fraction.

En somme, les résultats de Bailey et Lorand sont compatibles avec l'idée que la conversion fibrinogène → fibrine résulte d'une protéolyse. Nous savons maintenant que cette conversion s'accompagne de deux processus chimiques bien définis : un changement notable dans le nombre et la nature des résidus terminaux et l'ablation d'une partie (d'ailleurs vraisemblablement infime) de la molécule de fibrinogène. Cette ablation permet-elle, comme le suggère Lorand (26), l'association bout à bout des molécules pour former des fibres? L'hypothèse est attrayante. Mais elle reste à démontrer.

## LA CONVERSION CHYMOTRYPSINOGÈNE→CHYMOTRYPSINE

Les remarquables travaux de Northrop et Kunitz (48) nous ont appris que deux protéines inactives sécrétées par le pancréas (le chymotrypsinogène et le trypsinogène) sont capables d'être ultérieurement activées en donnant naissance à deux enzymes protéolytiques, la chymotrypsine et la trypsine. Ces enzymes sont eux-mêmes des protéines qui se différencient de leurs précurseurs par le pouvoir d'hydrolyser d'autres protéines. Deux groupes de chercheurs (*) ont récemment entrepris de déterminer de façon systématique les modifications qu'éprouve la structure des molécules au moment de l'apparition de l'activité enzymatique.

En 1935, Kunitz et Northrop (49) ont montré que le chymotrypsinogène cristallisé est activé par la trypsine. Les conditions d'activation choisies par ces auteurs semblent d'ailleurs assez particulières. Les quantités de trypsine sont si faibles que le traitement dure plus de 24 heures (activation lente). Au bout de ce laps de temps, on cristallise un enzyme protéolytique, la chymotrypsine-α, avec un rendement d'environ 50 %. Quand on abandonne cette première chymotrypsine dans des conditions favorisant en principe son autolyse, ou bien quand on laisse simplement vieillir les eaux-mères de la cristallisation, on obtient deux nouveaux types de cristaux actifs auxquels Kunitz (50) a donné le nom de chymotrypsines β et γ. Ces deux enzymes semblent avoir la même activité spécifique que l'enzyme-α.

Les faits mis en lumière par Northrop et Kunitz acquirent d'emblée un tel caractère classique, que pendant 15 années personne n'eut l'idée d'adopter d'autres conditions pour l'activation. Ces conditions sont pourtant arbitraires et certainement fort éloignées des conditions physiologiques. Ce fut le mérite de Jacobsen (14) d'avoir essayé en 1949 d'activer le chymotrypsinogène par des quantités de trypsine plus fortes (activation rapide). Les résultats furent d'ailleurs surprenants puisque les liquides obtenus manifestèrent vis-à-vis du lait un pouvoir coagulant supérieur à celui de l'enzyme-α cristallisé. L'auteur danois avait donc découvert que le chymotrypsinogène est capable d'engendrer plusieurs chymotrypsines et que, par conséquent, l'activité chymotrypsique n'est pas réservée à un seul type moléculaire. Toutes ces chymotrypsines doivent évidemment posséder une structure identique dans un large secteur de leur molécule puisqu'elles dérivent de la même protéine-mère. Certains détails structuraux doivent néanmoins être différents puisque l'activité est elle-même différente. En d'autres termes, nous nous trouvons ici dans une situation analogue à celle de tout à l'heure, avec cet élément supplémentaire d'intérêt qui s'attache aux protéines physiologiquement actives : quand on fait varier les quantités de trypsine, le chymotrypsinogène engendre une famille de chymotrypsines, comme l'ovalbumine engendre une famille de plakalbumines quand on fait varier les quantités de subtilisine.

Sur la foi d'études cinétiques, d'ailleurs très complètes, Jacobsen (14) émit en outre l'hypothèse que le chymotrypsinogène est converti par la trypsine en un enzyme primaire très instable, la chymotrypsine-π, qui donne ultérieurement naissance, soit à un enzyme-δ grâce à une seconde attaque trypsique (activation rapide), soit à l'enzyme-α par autolyse ou dégradation spontanée (activation lente). Correcte dans ses grandes lignes, cette hypothèse doit cependant être modifiée sur certains points de détail (voir plus loin).

Quand on passe en revue les diverses valeurs successivement attribuées au poids moléculaire de la chymotrypsine-α, on s'aperçoit que les premières sont très supérieures à celles trouvées pour le chymotrypsinogène. Cette situation assez absurde s'explique d'ailleurs de façon simple par l'aptitude de l'enzyme-α à former réversiblement un dimère. On admet maintenant (51) que les deux protéines (ainsi que les chymotrypsines β, γ et δ) possèdent un poids moléculaire analogue de l'ordre de 23-27 000. L'activation résulte donc de changements relativement minimes sur le plan moléculaire. Mais ces changements peuvent-ils être assimilés à une dégradation et cette dégradation résulte-t-elle de la rupture de liaisons peptidiques au sein du zymogène? Trois arguments semblent le suggérer : le fait que la trypsine, facteur déterminant de l'activation, est un enzyme protéolytique classique, l'augmentation du nombre des groupes carboxyle et aminés titrables et la production d'azote non-protéique. Ces arguments ne sont malheureusement pas

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(*) Je tiens à remercier ici de façon toute particulière Dr. Neurath d'avoir bien voulu m'autoriser à discuter ici ses propres résultats en même temps que ceux de mon groupe. Il nous a paru souhaitable de discuter ces résultats ensemble afin qu'ils acquièrent leur pleine signification.

plus déterminants que dans le cas de la plakalbumine. Les deux derniers ont même une signification plus aléatoire encore car les protéines-filles sont ici des enzymes protéolytiques qui peuvent s'autolyser pendant l'activation. Notons, en outre, sur un plan plus général que l'absence d'azote non-protéique ne signifie nullement que le processus étudié n'est pas protéolytique. Des liaisons peptidiques peuvent en effet être rompues sans que des peptides soient libérés. C'est pourquoi, bien que Butler (52) et Jacobsen (14) aient décelé une nette augmentation du nombre des groupements -COOH et $-NH_2$ titrables pendant l'activation lente et rapide, bien que d'autre part des quantités très notables d'azote non-protéique aient été trouvées dans les liquides activés (49, 14), on a hésité jusqu'à ces dernières années à prendre nettement parti. Ces hésitations étaient d'ailleurs tout à fait compréhensibles. On trouvera par exemple dans la figure 6 les courbes de l'azote non-protéique en fonction du temps. Le brusque changement de pente que ces courbes subissent au moment où l'activité atteint son maximum pourrait faire croire à une formation assez massive de peptides spécifiques. En fait, il n'en est rien (voir plus loin). Le peptide spécifique de l'activation apparaît au contraire avec un certain retard. L'azote non-protéique initial est donc vraisemblablement non-spécifique Les progrès techniques réalisés dans l'étude de la structure protéique ont heureusement permis depuis 1951 (53) d'attaquer le problème de façon plus directe et plus satisfaisante.

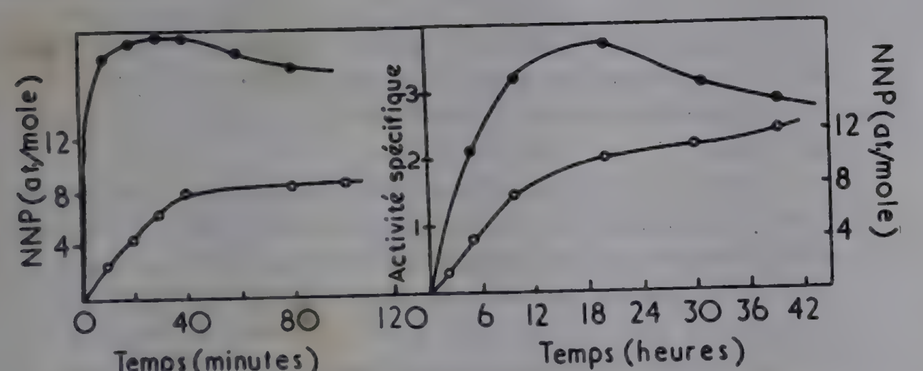


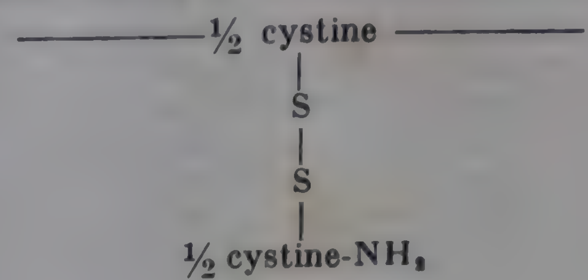

FIG. 6. — Activité spécifique et formation d'azote non-protéique, d'après Rovery *et al.* (54). A gauche : activation rapide ; à droite : activation lente.

○ azote soluble dans l'acide trichloracétique à 5 %,
● activité spécifique mesurée vis-à-vis de l'ester éthylique de la L-acétyltyrosine.

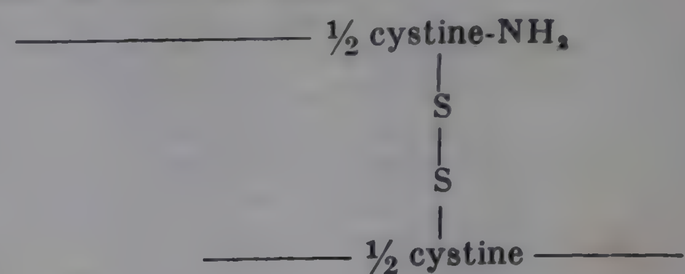
### *Résidus terminaux du chymotrypsinogène et de la chymotrypsine-α*

L'étude comparée des résidus terminaux de l'ovalbumine et des plakalbumines n'a pas permis jusqu'ici de se faire une idée très nette du mécanisme de la conversion. Cette étude a été au contraire très fructueuse dans le cas du chymotrypsinogène et des chymotrypsines. On a tout d'abord annoncé que le chymotrypsinogène est dépourvu de résidus N-terminaux (53, 55, 56) et qu'il ne libère aucun amino acide au contact de la carboxypeptidase (57). On pouvait donc le tenir à première vue pour cyclique. Mais Bettelheim (58) a montré un peu plus tard que la protéine contient un demi-résidu de cystine N-terminale (*). Ce demi-résidu n'est pas en position latérale (59) (fig. 7*a*). Il se trouve donc vraisemblablement placé à l'extrémité d'une chaîne (fig. 7*b*). La chaîne contient-elle un résidu C-terminal non-réactif vis-à-vis de la carboxypeptidase ou se termine-t-elle en boucle (60)? Cette importante question n'est pas encore élucidée.

*a*) Position latérale :




*b*) Position en bout de chaîne :




ou :

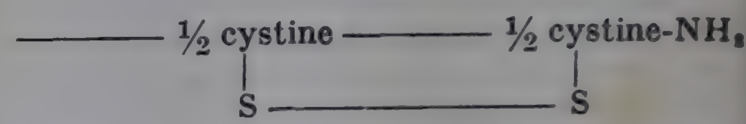



FIG. 7. — Diverses positions possibles pour la demi-cystine N-terminale du chymotrypsinogène.

Quoi qu'il en soit, la chymotrypsine-α contient deux résidus N-terminaux supplémentaires (l'*iso*leucine et l'alanine (53, 55, 56)) et, vraisemblablement aussi, deux résidus C-terminaux accessibles à la carboxypeptidase (la leucine et la tyrosine (57)). Ces résultats (*) rendent évidemment très probable la nature protéolytique du phénomène d'activation. Sans la rupture de certaines liaisons peptidiques au sein du chymotrypsinogène, on ne comprendrait pas en effet comment les résidus terminaux nouveaux aient pu se former (**).

---

(*) Ce résidu n'est décelable que si l'on oxyde préalablement la protéine par l'acide performique.

(*) Certains auteurs ont récemment publié des résultats différents. D'après Sorm, Keil et Rychlik (61), la phénylalanine (au lieu de l'*iso*leucine) serait en position N-terminale dans la chymotrypsine-α. Il s'agit vraisemblablement d'une erreur due à la présence de DNP-*iso*leucylvaline dans les hydrolysats de DNP-chymotrypsine-α. Le comportement chromatographique de ce peptide est tout à fait analogue à celui de la DNP-phénylalanine, sauf pendant la chromatographie sur papier en phase aqueuse (62). D'autre part, Akabori *et al.* (63), sur la foi de résultats obtenus par hydrazinolyse, ont cru pouvoir annoncer que le chymotrypsinogène et la chymotrypsine-α contiennent trois résidus C-terminaux identiques (glycocolle, alanine, acide aspartique). Bien que les performances de la carboxypeptidase ne soient elles-mêmes pas dépourvues d'ambiguïté, ces résultats sont tellement peu compatibles avec ce que nous savons maintenant du mécanisme de l'activation, que leur exactitude semble réellement douteuse.

(**) Par souci de brièveté, nous signalerons simplement ici que les chymotrypsines β et γ possèdent les mêmes résidus terminaux que l'enzyme-α (64, 57). Les différences de structure existant éventuellement entre ces trois enzymes ne sont donc pas décelables de cette façon.

*Mécanisme de l'activation rapide du chymotrypsinogène*

Nous avons vu que l'activation rapide du chymotrypsinogène engendre vraisemblablement une ou plusieurs chymotrypsines différentes de la chymotrypsine-α. En fait, les protéines formées au cours de cette activation contiennent bien l'*iso*leucine N-terminale de l'enzyme-α Mais elles sont dépourvues d'alanine N-terminale (65). Sitôt après cette observation fondamentale, une nouvelle chymotrypsine a été cristallisée sous forme de son dérivé di*iso*propylphosphorylé (66). Elle contient de la leucine C-terminale comme l'enzyme-α, mais elle est dépourvue de tyrosine C-terminale (66). Tout semble donc se passer comme si l'apparition des résidus d'*iso*leucine et de leucine était directement liée au processus d'activation puisqu'elle déclenche dans tous les cas la formation de chymotrypsines. L'apparition des résidus d'alanine et de tyrosine semble correspondre par contre à un processus secondaire et particulier à la génèse de l'enzyme-α. Loin d'être par lui-même activateur, ce dernier processus paraît diminuer la capacité d'activation du zymogène (tableau I).

formation de l'enzyme-α exige pour sa part une ou plusieurs ruptures supplémentaires faisant apparaître de la tyrosine et de l'alanine en position terminale. Cette image, encore bien schématique, a été confirmée et précisée de diverses manières.

Dans un très brillant travail, Bettelheim et Neurath (67) ont en effet montré par électrophorèse à pH = 4.97 que l'activation du chymotrypsinogène implique le remplacement progressif du pic de cette protéine par un pic plus lent. Mais la formation du pic lent est en retard sur l'activation (tableau II). On est donc forcé d'admettre que le pic rapide contient lui-même une chymotrypsine dont la mobilité est égale à celle du zymogène. Comme d'autre part l'ablation du peptide X-base peut être rendue responsable de la diminution de la mobilité en milieu acide, il apparaît vraisemblable que le pic lent est l'enzyme-δ et que le pic rapide est un mélange en proportions variables de zymogène et d'enzyme-π. Quand les conditions d'activation sont telles que le pic rapide est prédominant, le mélange contient d'ailleurs de fortes quantités d'*iso*leucine-N-terminale mais il est dépourvu de résidu C-terminal accessible à la carboxypeptidase.

TABLEAU I

*Résidus N-terminaux apparus pendant l'activation rapide et lente*

| | Activation rapide | | Activation lente | |
|---|---|---|---|---|
| | | | 40 heures | |
| | 10 min. | 3 h. | en l'absence de $(NH_4)_2SO_4$ | en présence de $(NH_4)_2SO_4$ |
| Activité spécifique | 3.7 | 3.9 | 3.2 | 1.5 |
| Résidus N-terminaux | | | | |
| *Iso*leucine | 1.00 | 0.95 | 1.10 | 0.5 |
| Alanine | 0.0 | ~ 0 | 0.2 | 1.2 |
| Thréonine | — | 0.2 | 0.4 | 0.1 |
| Autres résidus | 0.0 | 0.0 | 0.0 | 0.0 |

D'après Rovery *et al.* (65, 54).

Mais ces considérations ne doivent pas nous faire oublier que l'activation est due à la trypsine, laquelle manifeste une spécificité très étroite vis-à-vis des liaisons basiques. Comme l'ont fait très justement remarquer Gladner et Neurath (57), on devrait donc en toute logique s'attendre à ce que la première chymotrypsine possède un résidu C-terminal de lysine ou d'arginine. Le fait de trouver un résidu C-terminal de leucine suggère que notre enzyme cristallisé ne dérive pas directement du zymogène et qu'il correspond par conséquent à l'enzyme-δ de Jacobsen. Ce serait alors l'enzyme-π qui contiendrait le résidu C-terminal basique. L'image suivante, proposée dans l'une de nos communications préliminaires (66), commence ainsi à se dégager : l'activation proprement dite, c'est-à-dire la conversion du chymotrypsinogène en chymotrypsine-π est due à la rupture par la trypsine d'une liaison X-base. Dans une deuxième phase, la chymotrypsine-π perd un peptide X-base grâce à la rupture d'une liaison leucyl-X et se convertit en enzyme-δ. La

TABLEAU II

*Corrélations entre le degré d'activation et la conversion du pic rapide en pic lent*

| Durée de l'activation (minutes) | Activité estérasique (% de la valeur maximum) | Conversion du pic « rapide » en pic « lent » (%) |
|---|---|---|
| 5 | 55 | 9 |
| 15 | 76 | 24 |
| 30 | 93 | 64 |
| 90 | 100 | 100 |
| 1200 | 93 | 100 |
| 30 (*) | 81 | 3 |
| 70 (*) | 99 | 12 |
| 340 (*) | 100 | 21 |
| 1620 (*) | 99 | 48 |

(*) Essais en présence de β-phénylpropionate.
D'après Bettelheim et Neurath (66).

Quand, au contraire, le pic lent prédomine, on trouve à la fois de l'*iso*leucine N-terminale et de la leucine C-terminale (*).

*Identification du peptide X-base*

L'étude systématique des peptides mis en liberté pendant l'activation rapide du chymotrypsinogène a été entreprise simultanément par le groupe de Neurath et par le nôtre. L'un de ces groupes a utilisé la chromatographie sur résine (68) ; l'autre, la chromatographie sur papier et sur résine ainsi que la chromatographie des DNP-peptides (54, 69). Les résultats semblent d'ailleurs tout à fait concordants. Ils suggèrent que l'activation rapide libère un seul peptide spécifique, la sérylarginine (fig. 8).

La figure 9 donne en outre une représentation graphique de l'ensemble des réactions qui se produisent pendant l'activation rapide. Comme on le voit, les proportions de l'*iso*leucine N-terminale suivent fidèlement les variations du degré d'activation. C'est qu'en effet cette *iso*leucine appartient à la fois à l'enzyme-$\pi$ et à l'enzyme-$\delta$. Son apparition va donc de pair avec l'activation. Au contraire, la courbe de la sérylarginine est nettement décalée. C'est qu'elle correspond à une étape ultérieure du phénomène, laquelle n'est pas une activation mais bien la conversion d'un enzyme ($\pi$) en un autre enzyme ($\delta$) dont l'activité spécifique est analogue (**). En somme, la courbe de *iso*leucine N-terminale est une bonne expression de l'activation et la courbe de la sérylarginine traduit la cinétique de la conversion $\pi \rightarrow \delta$. Cette conversion est bien, comme les résultats de Bettelheim et Neurath le faisaient prévoir, ralentie par la présence de β-phényl propionate.

Tous ces résultats permettent de préciser notablement nos conceptions antérieures puisque le peptide X-base est en fait la sérylarginine. Nous pouvons donc dire que l'apparition de l'activité chymotrypsique et la rupture par la trypsine d'une liaison arginyl*iso*leucine au sein du chymotrypsinogène sont deux phénomènes simultanés. L'enzyme primaire $\pi$ perd ensuite la séquence sérylarginine grâce à la rupture vraisemblablement auto-

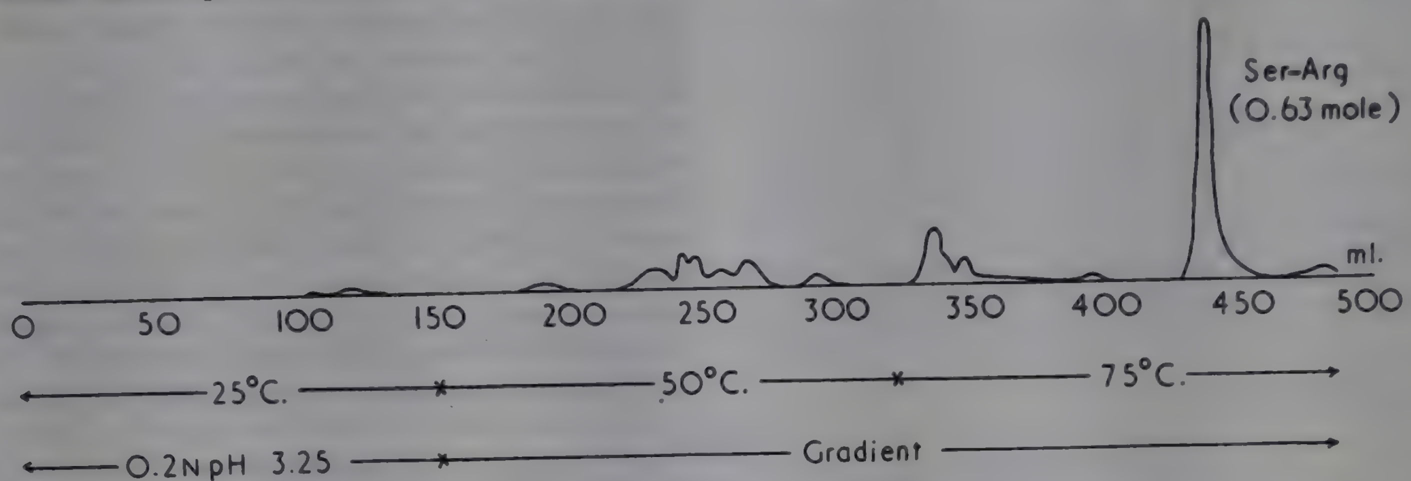



FIG. 8. — Chromatographie sur Dowex-50(× 4) des peptides engendrés par l'activation rapide, d'après Rovery, Poilroux et Desnuelle (70). Colonnes de Doxew-50(×4) de 100 cm. de hauteur et de 0.9 cm. de diamètre. Technique de Moore et Stein (71) utilisant un gradient de pH et de force ionique. Volume de la chambre de mélange : 300 ml. A) Tampon : 0.2 N, pH = 3.25. B) Tampon : 2 N, pH = 5.19. Fractionnement par ml. L'hydrolyse de la substance formant le pic principal fournit 1.2 mole d'arginine pour 1 mole de sérine. La sérine est N-terminale (technique de Sanger). Conditions d'activation : chymotrypsinogène 10.6 × $10^{-4}$ M ; trypsine 0.26 × $10^{-4}$ M. pH = 7.6. Durée : 1 h. Température 0° C. Activité spécifique, vis-à-vis de l'ester éthylique de la L-acétyltyrosine : 3.9.

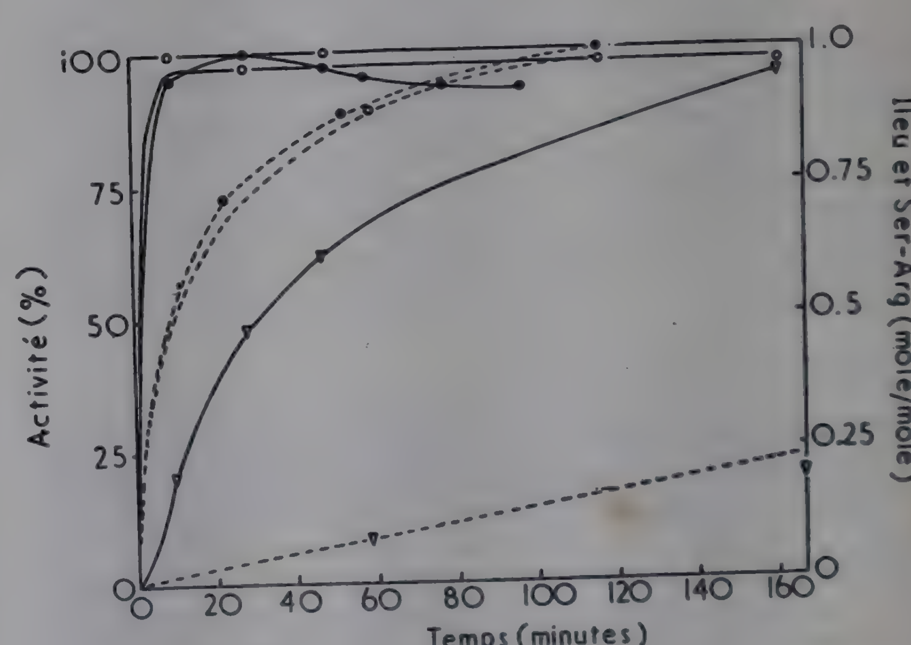


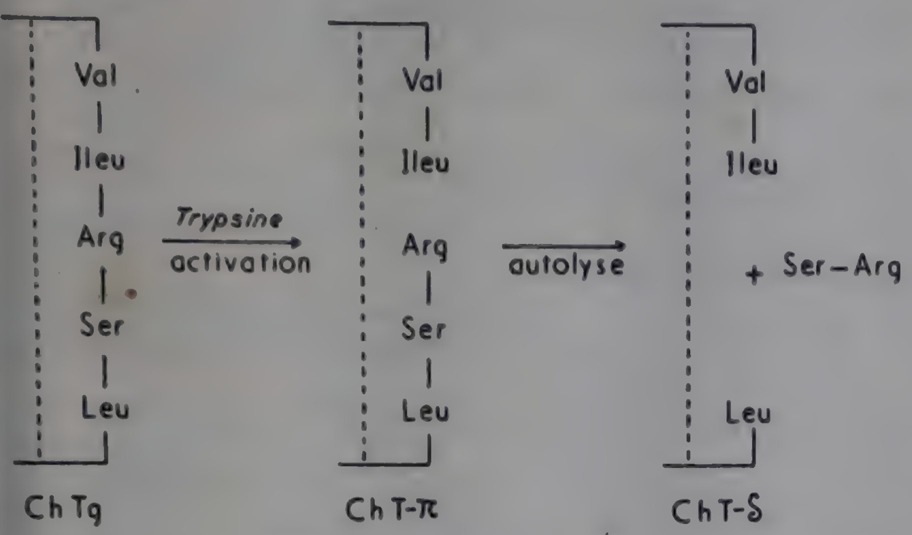

FIG. 9. — Cinétique comparée de l'activation et de la formation d'*iso*leucine N-terminale et de sérylarginine libre, d'après Rovery *et al.* (54). La détermination de l'*iso*leucine N-terminale et de la sérylarginine libre a été faite par colorimétrie des dérivés dinitrophénylés.

● activité spécifique estérasique (en % de l'activité maximum),
○ *iso*leucine N-terminale (mole/mole de zymogène),
▽ sérylarginine (mole/mole de zymogène),
——— activation en l'absence de β-phénylpropionate,
- - - - - activation en présence de β-phénylpropionate 0.1 M.

(*) En outre, Bettelheim et Neurath (67) ont réussi à montrer que la conversion $\pi \rightarrow \delta$ est vraisemblablement due, non à une nouvelle attaque trypsique comme le croyait Jacobsen (14) mais à une autolyse de l'enzyme-$\pi$. Les chiffres du tableau II indiquent en effet que le retard pris par la formation du pic lent est augmenté par la présence d'un inhibiteur de la chymotrypsine, le β-phénylpropionate.

(**) Bettelheim et Neurath (67) ont également suggéré que, contrairement à l'opinion de Jacobsen (14), les chymotrypsines $\pi$ et $\delta$ ont une activité analogue.

lytique d'une liaison leucylsérine. Cette perte ne semble pas affecter de façon sensible l'activité enzymatique de la molécule.




Le schéma établi sur ces bases n'est valable que pour l'activation rapide de Jacobsen. Il faut noter cependant que l'activation lente fait apparaître, non seulement l'*iso*leucine et la leucine terminale, mais aussi la séryl-arginine (54, 68, 69). Le mécanisme de l'activation est donc sans doute le même dans tous les cas, en ce sens qu'il implique toujours le démasquage du même centre actif. Néanmoins, l'activation lente provoque des ruptures supplémentaires dont nous cherchons à préciser la nature et l'origine. Ces ruptures, rappelons-le, sont défavorables puisqu'elles diminuent l'activité (sans toutefois la supprimer).

## ACTIVATION DU TRYPSINOGÈNE

Le trypsinogène est converti en trypsine soit par la trypsine elle-même, soit par un enzyme d'un pénicillium, soit enfin par un facteur mal défini de l'intestin, l'entérokinase (48). Etant donné que nous ne savons pas encore si ces diverses activations suivent la même route, il est nécessaire de préciser dès le début que nous allons étudier celle provoquée par la trypsine, c'est-à-dire l'activation autocatalytique (fig. 10). En présence d'ions calcium,

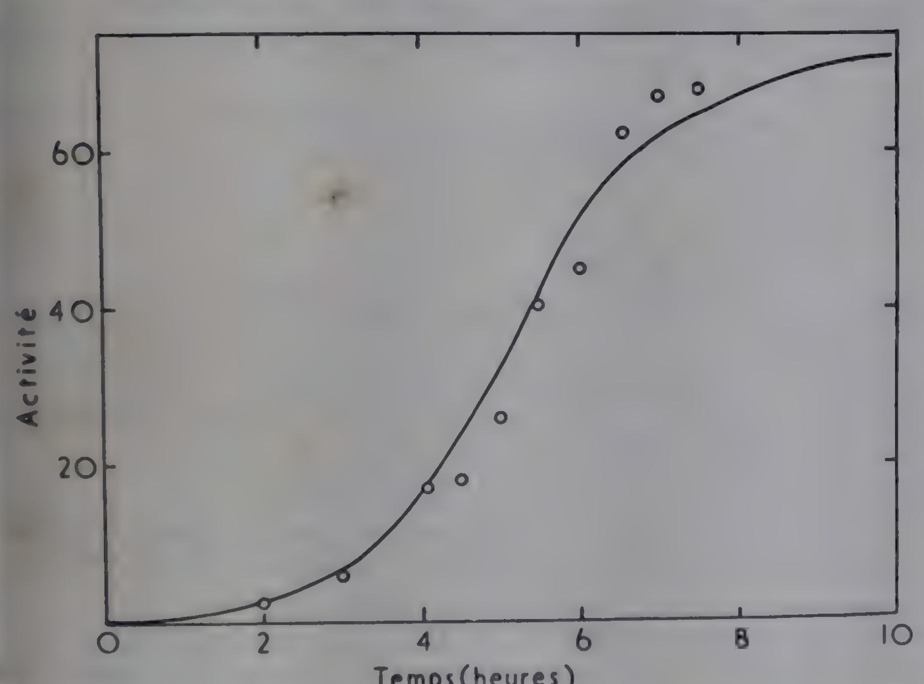



FIG. 10. — Activation autocatalytique du trypsinogène, d'après Kunitz et Northrop (72).

cette activation se produit selon la reaction simple suivante :

$$\text{Trypsinogène} + \text{trypsine} \rightarrow 2 \text{ trypsines}$$

Mais en l'absence d'ions calcium, des protéines inertes sont formées simultanément.

$$\text{Trypsinogène} + \text{trypsine} \rightarrow \text{trypsine} + \text{protéines inertes.}$$

La méthode de sédimentation-diffusion indique pour le trypsinogène un poids moléculaire de 23 700 (73). Les valeurs trouvées par diverses méthodes pour la trypsine s'échelonnent entre 20 500 et 24 800 (51). La variation, si elle existe, est donc trop faible pour être décelable par les techniques habituelles.

L'activation du trypsinogène n'implique pas, comme celle du chymotrypsinogène, l'apparition de résidus terminaux nouveaux, mais bien le remplacement d'un résidu terminal par un autre. Elle se rapproche donc davantage à cet égard de la conversion de la chymotrypsine-$\pi$ en chymotrypsine-$\delta$. On trouve en effet un résidu N-terminal de valine dans le trypsinogène et un résidu N-terminal d'*iso*leucine dans la trypsine (74) (*). L'hypothèse la plus simple consiste à supposer que la trypsine coupe une liaison X-*iso*leucine dans le trypsinogène et que la séquence N-terminale de cette molécule est scindée sous forme d'un peptide à valine N-terminale. Emise dès 1952 (74), cette hypothèse a reçu par la suite certaines confirmations d'ordre expérimental.

Davie et Neurath (76, 77) ont montré qu'un peptide spécifique se forme effectivement pendant l'activation (fig. 11). Ce peptide contient 1 mole de valine, 1 mole de

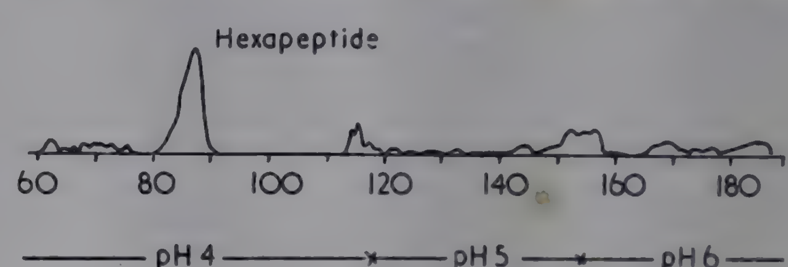



FIG. 11. — Chromatographie sur résine Dowex-50 des peptides engendrés pendant l'activation du trypsinogène, d'après Davie et Neurath (78). Colonnes de Dowex-50($\times$2) de 30 cm. de hauteur et de 0.9 cm. de diamètre. Analyse faite sur le filtrat trichloracétique d'une préparation de trypsinogène activé à 63 %.

lysine et 4 moles d'acide aspartique. Le résidu N-terminal est la valine. Les auteurs ont en outre supposé que la lysine est en position C-terminale puisque le peptide est libéré par la trypsine. Si tel est bien le cas, le peptide est : Val.Asp$_4$.Lys.

Par ailleurs, les séquences N-terminales du trypsinogène et de la trypsine ont été étudiées dans ce laboratoire (78, 79) au moyen de la technique classique de Sanger. Après hydrolyse partielle du DNP-trypsinogène,

(*) Sous l'influence de la carboxypeptidase, le trypsinogène et la trypsine ne libèrent par contre aucun amino acide (75). Bien que de faibles quantités de lysine semblent être engendrées quand les deux protéines sont préalablement dénaturées, nous préférons limiter la discussion aux événements qui se produisent sur le flanc aminé du zymogène au moment de son activation. Ces événements se traduisent par des changements précis de structure.

on peut isoler trois DNP-peptides : (DNP-Val.Asp.), (DNP-Val. Asp.Asp.) et un DNP-peptide contenant 1 mole de DNP-Valine, 1 mole d' (ε-DNP-lysine) et 4 moles d'acide aspartique. La similitude entre le peptide de Davie et Neurath et la séquence N-terminale du zymogène est frappante. On notera d'autre part que cette séquence ne se retrouve pas dans la trypsine, puisque l'enzyme commence par l'enchaînement Ileu.Val.Gly. Il n'est donc pas douteux que le peptide de Davie et Neurath est prélevé dans la séquence N-terminale du zymogène et que l'ablation de cette séquence se produit par conséquent pendant l'activation. Ce point très important étant acquis, il convient maintenant de se demander comment formuler la réaction. L'idée la plus simple est de considérer que le résidu C-terminal du peptide est relié dans le zymogène au résidu d'*iso*leucine qui deviendra N-terminal dans la trypsine et d'écrire par conséquent

$$\mathrm{Val} — \mathrm{Asp_4} — \mathrm{Lys} — \mathrm{Ileu} — \mathrm{Val} — \mathrm{Gly} —\ldots$$

Trypsinogène

$$\Big\downarrow \textit{Trypsine}$$

$$\underbrace{\mathrm{Val} — \mathrm{Asp_4} — \mathrm{Lys}}_{\text{Peptide}} \; + \; \underbrace{\mathrm{Ileu} — \mathrm{Val} — \mathrm{Gly} —\ldots}_{\text{Trypsine}}$$

D'après cette manière de voir, l'activation comporterait la rupture d'une liaison lysyl*iso*leucine, dont la parenté avec la liaison arginyl*iso*leucine du chymotrypsinogène est évidente. L'analogie pourrait d'ailleurs être poussée encore plus loin puisque, dans les deux cas, un résidu de valine est situé à droite du résidu d'*iso*leucine (56, 74). La trypsine posséde-t-elle une affinité particulière pour la structure Base-Ileu.Val et doit-on voir dans cette affinité l origine même du caractère limité de la protéolyse? Il est d'autant plus difficile de répondre à cette question que le schéma de l'activation du trypsinogène, contrairement à celui du chymotrypsinogène, relève encore de l'hypothèse à bien des égards. Pour en démontrer définitivement la validité, il faudrait être sûr que la lysine est C-terminale dans le peptide de Davie et Neurath et que ce peptide est le seul peptide spécifique formé pendant l'activation. Quoi qu'il en soit, la suggestion précédente mérite quelque attention.

En résumé, les protéolyses limitées auxquelles le chymotrypsinogène et le trypsinogène doivent leur activation ont pu être étudiées plus complètement que celles convertissant l'ovalbumine en plakalbumines et le fibrinogène en fibrine. Un heureux concours de circonstances en est la cause. Toutefois, l'image ainsi dégagée n'est certainement pas définitive. Elle devra dans l'avenir être retouchée et peut-être même modifiée de fond en comble. Son ambition n'est d'ailleurs pas d'expliquer le phénomène d'activation, mais bien plutôt de mettre en évidence certaines des réactions chimiques qui accompagnent cette activation.

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# The activation of trypsinogen and chymotrypsinogen

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(*Received* 11 *October* 1955)

I should like to take the opportunity offered by the organizers of the symposium to present such additional data on the activation of trypsinogen and chymotrypsinogen as either expand on the observations reported by Dr. Desnuelle or, by virtue of fact or interpretation, appear to be at variance with his views. The interest of two independent laboratories in the same problem has not only accelerated progress in this important field but has also aided in the critical and yet constructive evaluation of the results which have been obtained.

Conversion reactions involving the formation of active enzymes from inactive precursors, such as trypsinogen-trypsin, chymotrypsinogen-chymotrypsin, and as most recently studied by us, procarboxypeptidase-carboxypeptidase (1), are of unusual interest, since they represent the final steps in the formation of a biologically active protein and thus afford a close view of the relation of protein structure to biological activity. In particular, they raise the question whether the enzymatically active site is pre-existent in the zymogen molecule or whether it is formed by intramolecular rearrangement subsequent to the enzymatic cleavage of one or more strategically located peptide bonds. In view of a forthcoming, more detailed discussion of these problems (2), it may suffice to restrict the present consideration to the most salient points.

### *Trypsinogen*

There is little room for argument concerning the interpretation of the data relative to the tryptic activation of trypsinogen. The structure of the activation peptide determined by Davie and Neurath (3), *i.e.*

$$\text{Val-(Asp)}_4\text{-Lys},$$

seems to be in good agreement with the N-terminal sequence of trypsinogen as reported by Desnuelle and co-workers (4). More recently, the complete amino acid composition of trypsinogen and DIP-trypsin has been determined in our laboratory and, in general, the results appear to be in accord with the difference expected from the composition of the hexapeptide liberated during the tryptic activation of trypsinogen. The minimum molecular weight calculated from the amino acid analysis was 23 320 ± 280 for trypsinogen and 23 020 ± 340 for trypsin, both values being within the limits of the error of the methods previously employed for the determination of molecular weights by physical-chemical means. While the proposed activation scheme is in accord with all of these data, it remains to be determined whether activation by other enzymes, such as enterokinase or penicillium kinase, involves the opening of the same bonds or whether different forms of activated trypsinogen are produced in each case. It also remains for future work to elucidate the nature of the C-terminal portion of both trypsinogen and trypsin (3).

### *Chymotrypsinogen*

While the knowledge of the chemical structure of this protein is likewise incomplete, it is a tribute to the resolving power of the methods employed that it has been possible to pinpoint the amino acid sequence primarily affected by the activation process without any additional information on the composition of this zymogen (6, 7). It is of considerable interest that one of the five cystine residues (8) present in chymotrypsinogen occupies the N-terminal position (9). Since the protein is unreactive toward carboxypeptidase (10) and fails to yield any amino acid after hydrazinolysis (11), it appears attractive to suggest that the same cystine residue occupies both the N- and C-terminal position and that chymotrypsinogen consists of a single, closed polypeptide chain. (An open chain structure would require that the C-terminal amino acid contain an amide group). Contrary to the view expressed by Dr. Desnuelle, we believe that the presence of the N-terminal cystine group may prove to be a significant structural aspect of the activation process.

The molecular weight of chymotrypsinogen is now rather accurately fixed at 25 000 ± 1000, as determined by amino acid analyses (8), light scattering (unpublished experiments by Dr. J. Kraut) and by X-ray diffraction measurements of *iso*ionic crystals (private communication from Dr. J. C. Kendrew). Sedimentation equilibrium measurements likewise point toward this value, which is significantly higher than the value previously calculated from sedimentation analysis and diffusion measurements (12).

Several characterized derivatives of chymotrypsinogen have recently been prepared by Drs. Wilcox and Chervenka (13). In one derivative, all 13 ε-amino groups have been converted to guanidino groups; in another, one full α-amino group has been converted to a dithiocarbamate group; and in a third, all 14 amino groups have been acetylated. All of these derivatives can be fully activated by trypsin.

Elucidation of the complex nature of reactions attending the activation of chymotrypsinogen has required the application of a variety of methods, including N- and C-terminal group analysis, peptide analysis, electrophoresis, ultracentrifugation and enzymatic activity (esterase). Because of the simultaneous presence of two or more protein components in rapid activation mixtures, it was necessary first to establish conditions for the maximal enrichment of the component to be characterized (14, 15) and to determine quantitative component distribution. Prolonged moving boundary electrophoresis proved to be a sensitive tool, even though the mobility differences between chymotrypsinogen, π- and δ-chymotrypsins appeared to be relatively small (16). It required this type of analysis to find conditions for the preparation of nearly pure π- and δ-chymotrypsins, and to show that crystalline π-chymotrypsin (14, 17) was electrophoretically far more heterogeneous (14) than the activation mixture from which it was derived. Within the limits of experimental error, quantitative correlation was obtained between the electrophoretic components, identified as chymotrypsinogen, π- and δ-chymotrypsin, respectively, and the terminal groups appearing during activation (2, 15). Experiments involving the use of β-phenylpropionate (14, 15), an inhibitor for chymotrypsin, have provided strong evidence for the conclusion that contrary to the views of Jacobsen (18), the second step in the rapid activation, *i.e.* the conversion of π-chymotrypsin to the δ form, is catalyzed by chymotrypsin rather than by trypsin. The relatively large shift in electrophoretic mobility occurring during the π-δ conversion suggested the release of a basic peptide from the C-terminal portion of the molecule which has been isolated and identified as seryl-arginine (6, 15). The conclusion that the peptide was liberated during the conversion of π-chymotrypsin to the δ form was further strengthened by quantitative results in which the rates of formation and disappearance of the electrophoretic protein components during activation were compared to activity and the rate of formation of the dipeptide (15); the yield of peptide followed rather closely the curve describing the formation of δ-chymotrypsin. The enzymatic activity (esterase) was found to be proportionate to the concentration of chymotrypsinogen disappearing and all products of rapid activation were found to have the same specific activity. The scheme of rapid activation which we have proposed (6, 15) is identical to that just presented by Dr. Desnuelle and is predicated on the assumption that seryl-arginine is not a C-terminal sequence in chymotrypsinogen. This was proven by paper electrophoresis (6, 15), which showed that the spot corresponding to the dipeptide was absent when α-chymotrypsin was allowed to act on chymotrypsinogen or when the activation process was stopped at the π-chymotrypsin stage. The present scheme is likewise in full accord with the findings that both acetylated and guanidinated chymotrypsinogens can be fully activated (13).

A few words may be in order on the relation of the present findings to slow activation. It is to be expected that the protein components become more heterogeneous and the system more complex, the slower the rate and the longer the time of activation. This is clearly shown by end-group analyses (14) which have brought evidence for the existence of a hitherto unrecognized intermediate resulting on prolonged incubation of rapid activation mixtures. This intermediate, like α-chymotrypsin, contained both C-terminal tyrosine and leucine but was devoid of the N-terminal alanine. In view of these findings it appears unlikely that alanine and tyrosine occupy adjacent position (17) in the chymotrypsinogen molecule. The heterogeneity of slow activation mixtures and of the crystalline proteins obtained from them is also indicated by electrophoretic analysis (2, 16).

### *Mechanism of activation*

We should, finally, like to make certain observations on the structural implications of the findings reported today both by Desnuelle and by ourselves. It seems abundantly evident that in the case of both trypsinogen and chymotrypsinogen, the splitting of a single peptide bond suffices to convert the zymogen into the active form. This step is in both cases catalyzed by trypsin and opens a bond between a basic amino acid residue and an *iso*leucyl-valine sequence. The activating enzyme operates with a high degree of selectivity and restraint since in both enzymes there are approximately sixteen bonds which, on the basis of specificity, are potentially susceptible to hydrolysis by trypsin. In the case of trypsinogen, the bond opened during activation is situated near the amino end of the single peptide chain of this protein. It might be particularly susceptible to hydrolysis, as electrostatic repulsion between the four adjacent aspartic acid residues may keep the N-terminal sequence of trypsinogen in an extended configuration, whereas the remainder may be tied up in the folded structure and is thus protected against proteolysis. This interpretation is in accord with the views of Linderstrøm-Lang and Schellman (18), who propose that in a hydrogen-bonded helix all peptide bonds are resistant to enzymatic hydrolysis. In the case of chymotrypsinogen the exact position of the single peptide bond opened during conversion to the π form is not known, though its immediate environment is well characterized. Since, however, the dipeptide, seryl-arginine, is liberated only in the second step of the activation process, it is evident that the liberation of the dipeptide is a coincidental, rather than a functional, event.

Lastly, I should like to refer to very recent experiments (19), which suggest that the activation of chymotrypsinogen (and of trypsinogen), is accompanied by rather profound structural changes in the molecule, as evidenced by measurements of optical rotation. It was found that during the rapid activation of chymotrypsinogen, the specific levo-rotation of chymotrypsinogen decreases, in contrast to the increases in rotation usually encountered during denaturation. The change in rotation was proportional to the appearance of enzymatic activity (esterase). Since no further changes in rotation occurred during the subsequent conversion of π-chymotrypsin to the δ form, the result suggested that the cleavage of the single bond between arginine and *iso*leucine releases the internal strain in the polypeptide chain of the molecule and permits its conversion to a more tightly folded configuration. Qualitatively, similar findings have been obtained with trypsinogen, and these and other results will be reported in detail elsewhere. Since, as previously mentioned, trypsinogen can be activated also by enterokinase and by penicillium kinase, and chymotrypsinogen by a protease from *B. subtilis* (20), it is to be expected that the investigation of these activation processes by methods similar to those reported today will aid in the evaluation of the present conclusions and expand on their significance.

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# The biosynthesis of peptides and proteins (*)

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(*Received* 4 *April* 1955)

In the present state of the problem of the mechanism of peptide and protein synthesis an hypothesis is needed at the level of reaction mechanism in terms of chemical formulas. Very probably the clearer and the more detailed we may attempt to make the picture now the more clearly and the more definitely will it be demonstrated to have been wrong. But that will not be anything to be ashamed of.

The present review is such an attempt. The hypothesis is as follows. There are, viewed in the large, three steps in the synthesis of proteins. The first step is activation of the carboxyl group of free amino acids. The second step is transport of the amino acids so activated to a nucleic acid template where they are arranged in a specific sequence. On this template the amino acids are linked by peptide bonds and then the protein molecule is peeled off the template, this is the third step. Either in the course of its peeling off the template or shortly afterwards the specific coiling of the protein occurs spontaneously governed by its amino acid pattern. Or it is modified to another stable pattern by, for example, an agent such as an inducer in the formation of an adaptive enzyme, or an antigen in the formation of an antibody. Small peptides and quasi-peptides having, as it were, only a rudimentary amino acid pattern, certain features of protein synthesis are telescoped or missing in their synthesis.

Carboxyl groups are activated in a number of different types of enzymatic reactions. In the hypothesis to be presented here only the ATP-dependent carboxyl group activation is envisaged. Other types of carboxyl group activation are mediated by proteases, peptides and transferases and have been called replacement or transfer reactions. Their essential feature is conservation of some of the energy of the bond which is cleaved by activation of the carboxyl group of the cleavage product, presumably by combination with the enzyme; the enzyme may be displaced by water, ammonia, hydroxylamine, hydrazine, or the amino group of another amino acid or peptide. We know nothing at present regarding the role of such transfer reactions in the biosynthesis of proteins and peptides; it seems likely that they do occur *in vivo.* It is certain they are not involved in the first step by which free amino acids are built into peptides and protein, where there is a net gain of peptide bonds (1). The picture to be presented of the mechanism of protein synthesis is an extension and elaboration of the mechanisms by which quasi-peptides, amides and small peptides are synthesized. The main features of these mechanisms are now clear. We shall consider them first.

### *Synthesis of hippuric acid*

The steps in the synthesis of hippuric acid are as follows :

(1) $\mathbf{E} + \mathrm{ATP} \rightleftarrows \mathbf{E}\text{-}\mathrm{AMP} + \mathrm{P} \sim \mathrm{P}$

(2) $\mathbf{E}\text{-}\mathrm{AMP} + \mathrm{HS\text{-}CoA} \rightleftarrows \mathbf{E}\text{-}\mathrm{S\text{-}CoA} + \mathrm{AMP}$

(3) $\mathbf{E}\text{-}\mathrm{S\text{-}CoA} + \mathrm{HOOC\text{-}C_6H_5} \rightleftarrows \mathrm{CoA\text{-}S\text{-}OC\text{-}C_6H_5} + \mathbf{E}$

(4) $\mathrm{CoA\text{-}S\text{-}OC\text{-}C_6H_5} + \mathrm{H_2N\text{-}CH_2\text{-}COOH} \rightleftarrows \mathrm{C_6H_5\text{-}CO\ NH\text{-}CH_2\text{-}COOH} + \mathrm{CoA\text{-}SH}$

$$\mathrm{C_6H_5\text{-}COOH} + \mathrm{H_2N\text{-}CH_2\text{-}COOH} + \mathrm{ATP} \rightleftarrows \mathrm{C_6H_5\text{-}CONH\text{-}CH_2\text{-}COOH} + \mathrm{AMP} + \mathrm{P} \sim \mathrm{P}$$

The synthesis of hippuric acid from benzoic acid and glycine represents a gain in free energy of the same order of magnitude as that of a dipeptide bond, 2500-4000 calories (2). Accordingly the enzymatic synthesis of hippuric acid must be coupled with a free energy donor; Cohen and McGilvery (3) and Borsook and Dubnoff (4) found that the reaction was ATP dependent. Chantrenne (5) demonstrated that CoA is required, and then Shachter and Taggart (6, 7, 8) proved that it is benzoyl-CoA which reacts with glycine to form hippuric acid. The steps by which benzoyl-CoA are formed are taken directly from the mechanism given by Jones, Lipman,

(*) The preparation of this article and the work of the author referred to here were aided by a contract between the Atomic Energy Commission, administered by the Office of Naval Research, United States Navy Department, and the California Institute of Technology, Division of Biology (NR 122107). It was also supported by a research grant from the National Institutes of Health, United States Public Health Service, and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

Hilz and Lynen (9) for the formation of acyl-CoA. Shachter and Taggart showed that the benzoyl radical may be replaced, in conjugation with CoA, by a variety of aliphatic and aromatic acyl groups, but the enzyme is specific for glycine, and named it, therefore, glycine N-acylase (7). The enzyme for the synthesis of the acyl-CoA is presumed to be identical with the fatty acid activating enzyme.

Benzoyl-CoA reacts non-enzymatically with hydroxylamine to form benzoyl hydroxamate.

$$C_6H_5\text{-}CO\text{-}S\text{-}CoA + H_2NOH \rightarrow C_6H_5\text{-}CO\text{-}HNOH + HSCoA$$

Hydroxamate formation is a mode of trapping activated carboxyl groups.

It will be noted that it was the carboxyl, not the amino group which entered into the quasi-peptide bond that was activated. The energy for its activation came from one of the pyrophosphate bonds of ATP conserved, in part, in transmission through the transfers.

$$\text{E-AMP} \rightarrow \text{E-S-CoA} \rightarrow \text{CoA-S-Benzoyl}$$

It is to be noted also that the ATP is cleaved to AMP and inorganic pyrophosphate.

*Synthesis of pantothenic acid*

Maas and Novelli (10) showed that a similar cleavage of ATP promotes the synthesis of pantothenic acid, although CoA does not appear to be involved ; the enzyme was obtained from *Escherichia coli*.

The overall reaction is :

$$\underset{\text{(pantoic acid)}}{HO\text{-}CH_2\text{-}\overset{CH_3}{\underset{CH_3}{C}}\text{-}CHOH\text{-}COOH} + \underset{(\beta\text{-alanine})}{H_2N\text{-}CH_2\text{-}CH_2\text{-}COOH} + ATP$$

$$\rightarrow \underset{\text{(pantothenic acid)}}{HO\text{-}CH_2\text{-}\overset{CH_3}{\underset{CH_3}{C}}\text{-}CHOH\text{-}CONH\text{-}CH_2\text{-}CH_2\text{-}COOH} + AMP + P \sim P$$

*Synthesis of glutamine*

$$HOOC\text{-}CHNH_2\text{-}(CH_2)_2\text{-}COOH + NH_3 + ATP$$
$$\rightarrow HOOC\text{-}CHNH_2\text{-}(CH_2)_2\text{-}CONH_2 + ADP + H_3PO_4$$

Speck (11) and Elliott (12) discovered enzyme systems that can synthesize glutamine from glutamic acid and ammonia according to the above reaction. The $\gamma$-carboxyl of glutamic acid is the only acceptor of the free base, which may be ammonia, hydroxylamine, hydrazine or methylamine. Elliott (13) and later Varner and Webster (14) purified the enzyme greatly. The synthesizing activity throughout the purification paralleled the transferase activity which may be represented as :

$$\gamma\text{-glutamo-}NH_2 + \dot{N}H_3 \rightleftarrows \gamma\text{-glutamo-}\dot{N}H_2 + NH_3$$

In spite of this parallel it is uncertain whether or not the enzyme locus is the same for the synthetase and transferase activities. The synthetase optimum pH is 7.5, it is activated more by $Mg^{++}$ than by $Mn^{++}$, and is activated by cysteine. The transferase optimum pH is at 6.75, it is activated more by $Mn^{++}$ than by $Mg^{++}$, is not activated by cysteine, requires ADP or ATP, and phosphate or arsenate. ADP is a better cofactor for the transfer reaction than ATP. AMP is ineffective in both reactions. The same enzyme also catalyzes an arsenate activated hydrolysis of glutamine, for which $Mg^{++}$ or $Mn^{++}$ are required and ADP, ATP is inhibitory.

Phosphorylated intermediates of glutamic acid have been sought, and no trace of any have been found. Varner and Webster (14) tested the following possibilities as first steps in the reaction.

(1) $$\text{E} + \text{ATP} \rightleftarrows \text{E-P} + \text{ADP} \rightleftarrows \text{E-ADP} + \text{P}$$

or :

(2) $$\left.\begin{matrix}\text{E-P}\\ \text{or :}\\ \text{E-ADP}\end{matrix}\right\} + \text{Glu} \rightleftarrows \text{E-Glu} + \left\{\begin{matrix}\text{P}\\ \text{or :}\\ \text{ADP}\end{matrix}\right.$$

If any of these alternatives were correct, in the presence of glutamate and ATP either $ADP^*$ or $P^*$ should exchange its $P^*$ with that of ATP. This was found not to be the case. Such exchange did occur if a catalytic amount of $NH_3$ was added. The reaction mechanism appears to be somewhat as follows :

(1) $$\text{E} + \text{ATP} + \text{Glu} \rightleftarrows \text{E}\begin{matrix}\diagup\,\text{ATP}\\ \diagdown\,\text{Glu}\end{matrix}$$

(2) $$\text{E}\begin{matrix}\diagup\,\text{ATP}\\ \diagdown\,\text{Glu}\end{matrix} + NH_3 \underset{Mn^{++}}{\overset{Mg^{++}}{\rightleftarrows}} \text{Glu-}NH_2 + \text{ADP} + \text{P} + \text{E}$$

When this reaction was carried out in $^{18}O$ labeled water, no $^{18}O$ was found in any of the participants in the reaction. This finding attests to the correctness of the mechanism postulated as far as it goes.

Reaction (2) alone is the transfer reaction. In it arsenate can replace P. The overall reaction is far over to the right in favor of glutamine, so that only a slight exchange of $P^*$ can be demonstrated.

With hydroxylamine the overall reaction is even farther over to the right than with ammonia. Accordingly hydroxylamine could replace ammonia in glutamine (the transfer reaction), and no transfer of $P^*$ or $ADP^*$ into ATP could be demonstrated.

Here again it is certainly the $\gamma$-COOH of glutamate which is activated. No CoA is involved. Unlike the syntheses of hippuric acid and pantothenic acid the cleavage products of ATP are not AMP and P-P but ADP and P.

Webster and Varner (15) have found a different but in all respects analogous enzyme for the synthesis of asparagine and asparaginylhydroxamate from aspartic acid and ammonia or hydroxylamine.

### *Synthesis of glutathione*

The enzymatic synthesis of glutathione from its constituent amino acids was achieved by Bloch and coworkers and the overall reactions were demonstrated to be as follows (16-22).

$$(1)\quad \text{Glu} + \text{Cys} + \text{ATP} \xrightarrow{K^+,\ Mg^{++}} \gamma\text{-Glu-Cys} + \text{ADP} + \text{P}$$

$$(2)\quad \gamma\text{-Glu-Cys} + \text{Gly} + \text{ATP} \xrightarrow{K^+,\ Mg^{++}} \gamma\text{-Glu-Cys-Gly} + \text{ADP} + \text{P}$$

The synthesis of each peptide bond uses one pyrophosphate linkage of ATP. ADP is inhibitory. Apart from ATP no other coenzyme appears to be required. No phosphorylated intermediates have been found.

In the synthesis of each peptide bond the first step is :

$$\mathbf{E} + \text{ATP} \rightleftarrows \mathbf{E}\text{-P} + \text{ADP}$$

This was established for the formation of the Glu-Cys bond by Webster and Varner (23) and for the Cys-Gly bond by Snoke (24). The evidence in both cases was the same. The enzyme was incubated with ATP and $AD^{32}P$ and the ATP became labeled. The sequence of events was, therefore,

$$(1)\qquad \mathbf{E} + \text{ATP} \rightleftarrows \mathbf{E}\text{-P} + \text{ADP}$$

$$(2)\qquad \mathbf{E}\text{-P} + AD^{32}P \rightleftarrows \mathbf{E} + AT^{32}P$$

$$(1)+(2)\qquad \text{ATP} + AD^{32}P \rightleftarrows \text{ADP} + AT^{32}P$$

With **E**-P established as the first step, Webster and Varner (23) established the next step to be :

$$\mathbf{E}\text{-P} + \text{Glu} \rightleftarrows \mathbf{E}\text{-Glu} + \text{P}$$

They incubated $P^*$ with the enzyme, glutamate and ATP and found that the ATP became labeled. The reaction sequence was therefore :

$$\mathbf{E} + \text{ATP} \rightleftarrows \mathbf{E}\text{-P} + \text{ADP}$$

$$\mathbf{E}\text{-P} + \text{Glu} \rightleftarrows \mathbf{E}\text{-Glu} + \text{P}$$

When cysteine was added less $P^*$ was incorporated into the ATP. The final step, therefore, is :

$$\mathbf{E}\text{-Glu} + \text{Cys} \rightleftarrows \mathbf{E} + \text{Glu-Cys}$$

If the reaction mechanism had been :

$$\gamma\text{-Glu-P} + \text{Cysteine} \rightleftarrows \gamma\text{-Glu-Cys} + \text{P}$$

then no transfer of $P^*$ into $ATP^*$ should occur in the absence of cysteine and addition of cysteine should increase the phosphate transfer. The reverse occurred (20).

It is interesting that the purified glutamine synthetase enzyme was found to catalyse the synthesis of γ-glutamylcysteine.

The reaction mechanism appears to be analogous for the synthesis of the Cys-Gly bond of glutathione; but the equilibrium is much further to the right.

$$\mathbf{E}\text{-P} + \text{Glu-Cys} \rightleftarrows \mathbf{E}\text{-Glu-Cys} + \text{P}$$

$$\mathbf{E}\text{-Glu-Cys} + \text{Gly} \rightleftarrows \text{Glu-Cys-Gly} + \mathbf{E}$$

From this scheme one would expect that when the enzyme is incubated with $P^*$, Glu-Cys and ATP that the ATP would become labeled. In the experiments of Snoke *et al.* (25) the result was negative. But Webster and Varner (26) using highly labeled $^{32}P$ did find a low degree of labeling of the ATP when the reaction mixture contained Glu-Cys but not otherwise. Either the equilibrium is very far over to the right or the intermediate is :

$$\mathbf{E}\begin{array}{l}\diagup \text{P}\\ \diagdown \text{Glu-Cys}\end{array}$$

which then reacts with Gly to give Glu-Cys-Gly—**E**+P.

The hydroxamate of Glu-Cys can be formed in this reaction, but 0.4 M $NH_2OH$ is required to be as effective as $10^{-3}$ M glycine.

No synthesis of glutathione by transfer reactions of the type :

$$\gamma\text{-Glu-Gly} + \text{Cys-Gly} \rightleftarrows \gamma\text{-Glu-Cys-Gly} + \text{Gly}$$

or :

$$\gamma\text{-Glu-Cys} + \gamma\text{-Glu-Gly} \rightleftarrows \gamma\text{-Glu-Cys-Gly} + \text{Glu}$$

could be demonstrated by Snoke *et al.* (25) with their purified enzyme. Fodor *et al.* (27) using a kidney enzyme did find a synthesis of glutathione to occur by a transfer reaction of the type :

$$\text{Cys-Gly} + \gamma\text{-Glu-Gly} \rightleftarrows \gamma\text{-Glu-Cys-Gly} + \text{Gly}$$

There is no utilization of ATP in this mode of glutathione synthesis. There are no data on the biological significance of this reaction.

### *Carboxyl group activation in the synthesis of protein*

In the five types of synthesis we have considered, in only one was CoA involved, in two ATP was cleaved to AMP and pyrophosphate and in three others to ADP and orthophosphate. All five had two features in common, it was the carboxyl group that was activated, and this activation was achieved at the expense of one or the other pyrophosphate bonds of ATP. The five syntheses differed in the form of the activated carboxyl group; it was linked to CoA, or directly to the enzyme, or in an enzyme-$PO_4$ complex. In no case was the activated carboxyl group a carboxyl-$PO_4$ of the free amino acid.

Also in all refashioning of new peptide or amide bonds by transfer reactions not involving cleavage of ATP, the evidence again is very strong that it is the carboxyl group which is activated by remaining attached to the enzyme. No activation of the amino group is required, it combines with the activated carboxyl group to reconstitute the peptide (or amide) bond simply by displacing the enzyme from the RCO-E linkage.

On the basis of analogy, therefore, the author has suggested that the first step in protein synthesis was activation of the carboxyl groups of free amino acids, utilizing for this purpose one of the pyrophosphate bonds

of ATP (28). In addition there was some supporting experimental evidence in the literature. Peterson and Greenberg (29) had found that ATP accelerated amino acid incorporation into proteins *in vitro* in an enzyme system that was composed of mitochondria plus a supernatant fraction of rat liver homogenate. Siekevitz (30) had found evidence of a soluble cofactor produced by mitochondria which stimulated amino acid incorporation *in vitro* into rat liver microsomes. He also observed some stimulation by ATP, and was of the opinion that ATP was involved in the formation of the soluble cofactor. Zamecnik and coworkers followed up the observations (31, 32) that the microsomes of the liver have the highest rate of amino acid incorporation of any other liver fraction and demonstrated that this incorporation requires ATP and a heat labile, non-dialysable constituent of the supernatant fraction of liver homogenate after it had been centrifuged at 100 000 *g* (33-35).

In Zamecnik's laboratory Hoagland (36) has described experiments which indicate that the heat labile, non-dialysable fraction of liver supernatant contains an enzyme which, with ATP, activates the carboxyl group of free amino acids. The following reaction mechanism was suggested :

$$\mathrm{E} + \mathrm{ATP} + \mathrm{HOOC\text{-}CHRNH_2} \rightleftarrows \mathrm{E\text{-}AMP\text{-}CO\text{-}CHRNH_2} + \mathrm{P\text{-}P}$$

The evidence published so far is the formation of hydroxamate. The mechanism given was :

$$\mathrm{E\text{-}AMP\text{-}CO\text{-}CHRNH_2} + \mathrm{NH_2OH} \rightarrow \mathrm{E} + \mathrm{HONH\text{-}CO\text{-}CHRNH_2} + \mathrm{AMP}$$

The main evidence presented in support of the conclusions drawn regarding carboxyl group activation was an increase in the hydroxamate color with iron when amino acids were added to a reaction mixture containing the enzyme, ATP and other necessary ingredients. It was also stated in the note in which this work appeared that a spot was obtained on a filter paper chromatogram corresponding to leucinehydroxamate. We have made similar findings with the supernatant after centrifugation at 100 000 *g* of lysed rabbit reticulocytes. At present we have reservations regarding both Hoagland's data and our own. In the colorimetric assay for hydroxamates (37) using iron in acid solution (pH 0.5-1.5) the color given by amino acid hydroxamate is very much less than that given by, for example, acet- or succinyl-hydroxamate. In both Hoagland's experiments and ours the increase in color due to amino acids was very small. In our experiments the addition of ATP increased the amount of hydroxamate color after one hour's incubation and this color was further increased by the addition of amino acids. At present we are engaged in an attempt to obtain more direct evidence by using labeled amino acids and carrier isolation of the labeled hydroxamate. The present, tentative, findings are positive.

If the interpretation now held very tentatively is confirmed, the findings will constitute direct evidence that amino acid incorporation is based on, and must be preceded by, activation of the carboxyl groups of amino acids. Given activated carboxyl groups of amino acids the amino groups of other amino acids need no activation for formation of peptide bonds.

Support for the view that the first step in the synthesis of protein is activation of the carboxyl groups of free amino acids by ATP can be found in the many observations that the formation of adaptive enzymes in micro-organisms depends upon the simultaneous presence of an energy source and free amino acids (38-47). This energy may be generated either aerobically or anaerobically (38, 46, 47). Repeatedly in reports of this work one reads the statement emphasized that free amino acids (or a nitrogen source readily convertible to amino acids), an energy source (or active metabolism) and the inducer of adaptation must be present together. Conversely inhibitors of respiration, and even more significantly, inhibitors of oxidative phosphorylation, thus inhibiting formation of ATP, promptly inhibit formation of adaptive enzymes (39, 42, 46). This is the situation also in micro-organisms (33-35). Gale and Folkes (45) showed that the same occurs in disrupted cells of *Staph. aureus*, *i.e.* in intracellular particles of these organisms.

In the earliest studies of protein metabolism in mammals, nearly a half century ago, it was found that when the amount of protein eaten tended to be short, carbohydrate had a sparing action on the amount of nitrogen lost from the body (48). Geiger (49, 50) and Munro (51) showed that, to exert this effect, the carbohydrate must be fed at the same or within a short time of the protein.

The interpretation is that during the feeding of carbohydrate there is an increased generation of ATP at the same time that the blood and tissue amino acid concentration is increased. Consequently the concentration of activated amino acids is increased and this concentration, all other things being equal, governs the rate of protein synthesis. The protein sparing action of carbohydrate means a faster rate of protein synthesis.

Table I shows the stimulating effect of carbohydrate metabolism on protein synthesis *in vitro*. These data were obtained with rabbit reticulocytes. The protein synthesized was mainly (90 % or more) hemoglobin. The cells contain some sugar initially and hence added carbohydrate became effective only after the sugar initially in the cells was used up, which was from 2 hours onwards. Even then the added sugar has no effect unless the conditions are suitable for a very high rate of protein synthesis, *i.e.* optimal amino acid mixture and an excess of iron. The high rate of protein synthesis accelerates the utilization of the original sugar in the cells and then, the data show, added sugar is necessary to maintain the high rate of protein synthesis.

It seems likely that the phenomenon known as the specific dynamic action of proteins is based on an increased rate of generation and utilization of ATP evoked by an increase in the concentration of amino acids in the blood and tissues. Shortly after a protein meal is eaten and for a few hours thereafter there is a considerable increase in metabolism; it may be as large as 25-30 %. This increase is three to five times as great as after eating an isocaloric quantity of carbohydrate or fat. The rise and subsequent fall in metabolism follows fairly closely the rise and fall of amino acid concentration in the blood and presumably in the tissues (54). There is increased

TABLE I.

*Effect of glucose on protein synthesis in rabbit reticulocytes* in vitro

| | Incorporation (counts/min./mg.) after | | | | Effect of glucose (counts/min./mg.) after | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 h. | 2 h. | 3 h. | 4 h. | 1 h. | 2 h. | 3 h. | 4 h. |
| Blank | 3.32 | 4.75 | 6.38 | 7.43 | | | | |
| + glucose | 3.12 | 5.03 | 6.89 | 7.72 | —0.20 | 0.38 | 0.51 | 0.29 |
| + amino acids | 14.59 | 22.94 | 25.78 | 26.01 | | | | |
| + glucose + amino acids | 15.01 | 26.15 | 34.05 | 36.84 | 0.42 | 3.21 | 8.27 | 10.83 |
| | | | | | (% of total incorporation) | | | |
| | | | | | 2.7 | 12.2 | 24.2 | 29.3 |

utilization of ATP in the increased activation of carboxyl groups, in increased urea formation and increased protein synthesis, and increased oxidative deamination. Hence the specific dynamic action of protein (55).

### *The question of peptide intermediates*

After the amino acids have been activated the next step is their transport in the activated state to the matrix on which the specific pattern of amino acids is formed, to the template as it has been called. No energy is gained by making peptides at this stage. The making of each peptide bond would require an activated carboxyl group. There would be needed for attachment to the template and for the making of the peptide bonds by which the protein is finally put together an activated carboxyl group on each peptide. There is much more flexibility for the making of many different amino acid patterns in doing so from activated single amino acids than from activated peptides.

If proteins are made directly from amino acids without the intervention of peptides, then, if the amino acids are labeled, the same amino acid should have the same specific activity at all loci in the protein molecule. If there are peptide intermediates one might expect some peptides to be in the cell before the advent of a labeled amino acid, containing the same amino acid unlabeled. In the synthesis of the protein, the peptide would go to form one portion of the molecule, the free labeled amino acid other portions. The result would be that until the preexisting peptide was largely used up there would be unequal labeling of an amino acid at different loci in the protein molecule. One may not generalize this argument by substituting the idea or the word 'intermediate' for 'peptide'. An 'intermediate' could be the activated amino acid.

Anfinsen and his collaborators found unequal labeling of amino acids in ovalbumin (56-58), insulin and ribonuclease (60). The inequality of labeling was greatest in the beginning and tended to grow less the longer the experiment. *In vivo* and *in vitro* experiments gave similar results (58).

Steinberg and Anfinsen (58) were of the opinion that their results were explained best by the postulate of different sized pools of preformed peptide intermediates. Labeled amino acids would first be incorporated into different sized pools of the hitherto unlabeled peptides. The specific activity of the amino acid would then differ from peptide pool to peptide pool, and when these peptides combined to form the protein the different segments corresponding to these peptides would contain the labeled amino acid at different specific activities.

The evidence against peptides playing such a rôle is as follows. This explanation calls for impossibly large amounts of preformed peptides (1). Repeated attempts to find such peptide intermediates, even in very small amounts, using labeled amino acids, have failed to find any.

In feeding experiments peptides or partial hydrolysates were found to be less effective than amino acids or complete hydrolysates in feeding animals (60, 61). Indeed many peptides were excreted unused. In the tissue culture experiments of Gerarde *et al.* (62) the greatest incorporation of labeled amino acids was obtained when the culture medium contained, instead of the embryo extract which contained proteins and peptides as well as amino acids, a mixture of 19 amino acids.

In rabbit reticulocytes *in vitro* the ratio of glycine incorporation into the heme and into the globin per residue of glycine over a wide range of rates of hemoglobin synthesis was found to be 1. This finding excludes the preexistence of any significant concentration of intermediates or precursors of heme or of globin more complex than free amino acids (63). Experiments on antibody formation and on the formation of adaptive enzymes in microorganisms argue against more complex precursors than free amino acids in the formation of such specific proteins (64-72).

The unequal labeling which led Anfinsen and his coworkers to envisage peptides as intermediates in protein synthesis has not been found by other workers under other experimental conditions. Thus Muir *et al.* (73) found, after giving labeled amino acids to rats and rabbits, that the terminal and non-terminal valine of hemoglobin had the same specific activity. Velick and coworkers compared the specific activities of the same amino acid in three enzymes with different rates of turnover in muscle after injection of labeled amino acids to rabbits. Eight amino acids were examined. If there were any significant amount of peptides one would expect

the specific activities of the same amino acid to be different in the three proteins. With all eight amino acids the ratio of the specific activities in the three enzymes was the same. The conclusion was that the three enzymes were formed from the same amino acid pool and that a given amino acid in a protein is replaced at the same rate in all of the positions in which it occurs (74, 75). Heimberg and Velick (75), found that the main incorporation, *i.e.* synthesis of new protein, occurred within the first 2 hours after injection of the labeled amino acid.

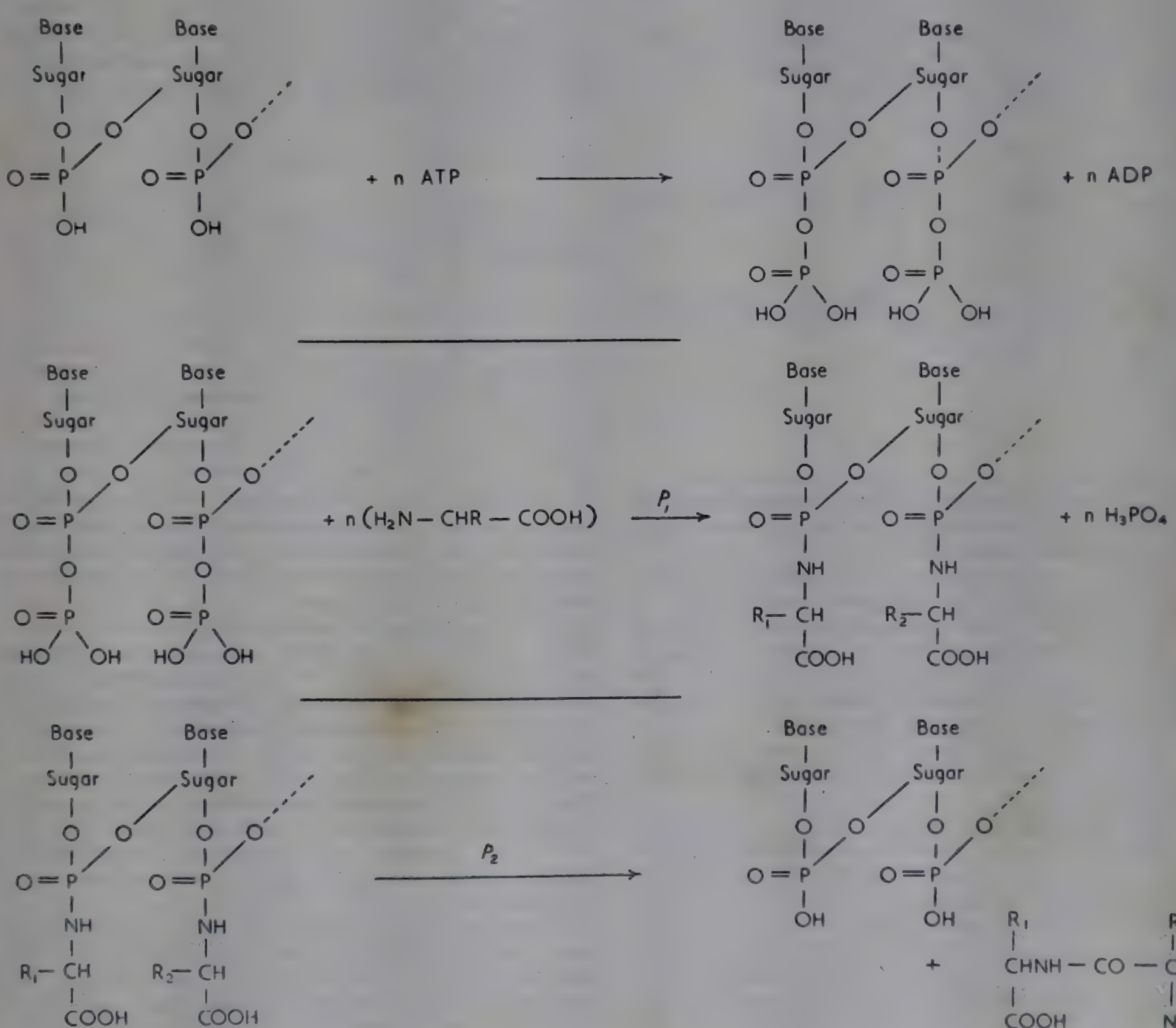
Work and his collaborators earlier had made comparisons such as those of Velick and coworkers on casein and whey proteins; and their findings were the same (76). Later (77) they isolated peptides from partially hydrolysed casein and compared the specific activities of a number of labeled amino acids common to them. They found that the same amino acid had the same specific activity in all the peptides, except the valine in one peptide. They concluded that casein is synthesized from free amino acids and that no peptides take part as such. Barry's findings were similar and he came to the same conclusion (78).

In our experiments on hemoglobin synthesis *in vitro* in rabbit reticulocytes a value of 1 was found for the molar ratio of incorporation of lysine and histidine per residue of amino acid in globin. This confirmed the conclusion drawn from the rates of glycine incorporation into heme and globin that there are no significant amounts of complex precursors (peptides) of globin. Muir *et al.* had come to the same conclusion earlier from their *in vivo* experiments (73).

If peptides participate in protein synthesis their existence must be very transient and the amount present at any time must be very small.

There can be no question of the fact of unequal labeling observed by Anfinsen *et al.*, nor of the observations where unequal labeling was not found. But the explanation must be other than significant amounts of peptide intermediates. A possible explanation will be given below.

Dounce's scheme :



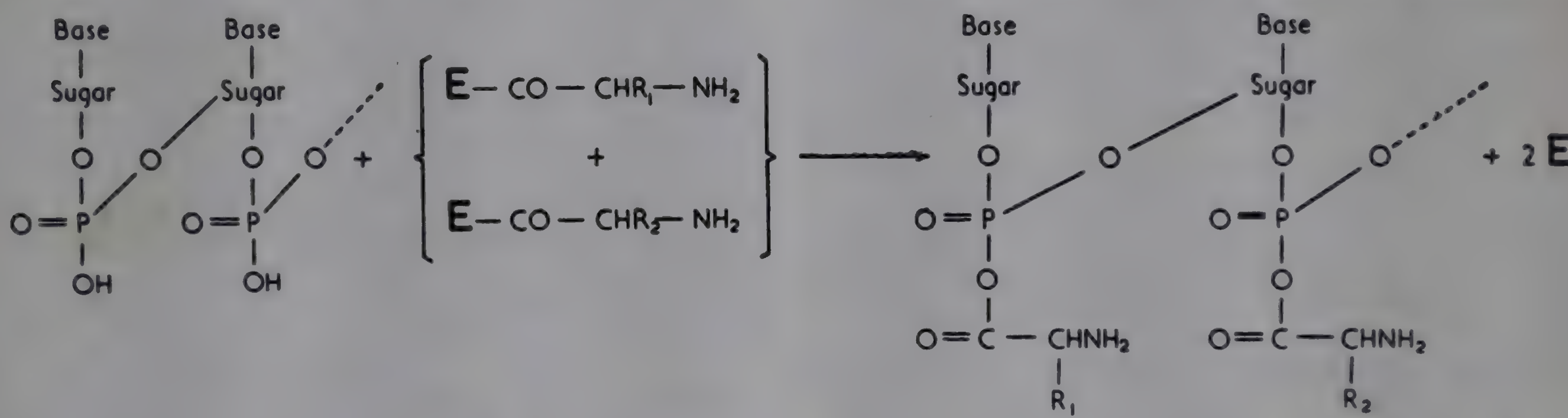
### *RNA the pattern forming template*

We have assigned the action of ATP to activation of the carboxyl groups of free amino acids by a special activating enzyme. Some have suggested that the activating enzyme is the template itself. Dounce (79) has been the most explicit proponent of this view. In his view ATP contributes the necessary energy by means of a phosphotransferase which transfers its terminal phosphate to the phosphate of nucleic acid, the net result is transfer of a pyrophosphate linkage from ATP to nucleic acid. The amino groups of amino acids then react to form amino-phosphate compounds on the nucleic acid, and for each amino group so combined the phosphate which came originally from the ATP is displaced and appears as inorganic phosphate. Another enzyme links the free carboxyl group of the adjacent amino acid to the phosphate bound-amino group to form a peptide linkage and the original phosphate of the nucleic acid.

One class of enzymes, $P_1$, mediates attachment of the amino acids to the nucleic acid; another, $P_2$, effects the formation of the peptide bonds and the concomitant liberation of the peptide chain from the template.

In our hypothesis the amino acids are attached to the phosphates of nucleic acid by their carboxyl groups.

The scheme envisaged is as follows :



Then as in Dounce's scheme another enzyme forms the peptide bonds thereby removing the amino acids involved from the nucleic acid template.

In our scheme some mechanism for transporting the activated amino acids to the template is called for. It might be the amino acid attached to the activating enzyme, or some smaller molecular weight carrier, a coenzyme or nucleotide. Whatever it may be the energy of activation is largely retained in the transfer from the activating enzyme to the template.

First the basic notion needs to be examined that nucleic acid and especially RNA, is so directly involved in protein synthesis.

More than a decade ago Brachet (80) and Caspersson (81-84) called attention to a mass of circumstantial evidence and adduced indirect experimental evidence implicating nucleic acids in protein synthesis. This evidence has been added to; for example, among the various cell fractions it is the particles with the highest concentration of ribonucleic acid (85), the microsomes, which incorporate amino acids into their proteins fastest (31-35). Similarly in the course of the development of reticulocytosis in rabbits there is a 30-40 fold increase in RNA and a roughly parallel increase in the ability to synthesize hemoglobin (and other proteins) the increase in DNA is only 2-3 fold (86-87). Caldwell *et al.* (89, 90) found in *Bac. lactis aerogenes* that the RNA content of the cell is approximately proportional to the rate at which the cell had actually been growing, *i.e.* to the rate, *inter alia*, of protein synthesis. They suggested that in the synthesis of protein the nucleic acid determines the amino acid pattern, and conversely the amino acid sequence of the protein determines the specific configuration of the nucleic acid; this picture was accepted by Dounce (79). Jeener (91) concluded from observations on experimentally induced variations in nuclear and cytoplasmic volumes of *Thermobacterium acidophilus* that protein synthesis was quantitatively related to that of RNA, and that its dependence on the presence of DNA is much more remote and indirect. In yeast the adaptive formation of galactozymase is inhibited by ultraviolet light; the action spectrum of the inhibition is that of nucleic acid and markedly different from that of protein (92).

Direct experimental evidence has now been found implicating RNA in protein synthesis. Gale and Folkes (93) showed that protein synthesis in the particles of a cell-free preparation of *Staph. aureus* was abolished by treatment with ribonuclease. Lester (94, 95) found the same in a cell-free preparation of *Microc. leisodeikticus*. The incorporation of labeled amino acids by onion root tips (96) and by rat liver microsomes (34) is inhibited by ribonuclease.

The dependence of protein synthesis on DNA is less direct than it is on RNA. Amino acid incorporation and protein synthesis can be demonstrated *in vitro* in the non-nucleated fractions of mammalian cells (31-35, 88, 97-99). Non-nucleated fragments of protozoa incorporate labeled amino acids into their proteins (97). Irradiation with X-rays (100-103) which inhibits synthesis of DNA does not inhibit RNA and protein synthesis to the same extent, or it may not inhibit the latter at all. At dosages of ultraviolet irradiation that inhibit DNA synthesis RNA and protein synthesis continue (71).

S-mustard stops growth in *E. coli* (*i.e.*, DNA synthesis) but not adaptive enzyme formation (105, 106). A mutant of *E. coli* which requires thymine, *i.e.* for DNA synthesis, in the absence of thymine and unable to multiply, could produce adaptive enzymes (107).

### *Relation between RNA synthesis and protein synthesis*

The mere presence of some RNA is not sufficient for protein synthesis (86, 96, 107). Mature rabbit erythrocytes contain about 40 mg. % of RNA (86) and yet they cannot synthesize protein. On the other hand there need be no net increase in RNA for extensive synthesis of protein (108); but in every case where there is rapid protein synthesis, turnover, *i.e.* synthesis of RNA is observed (45, 72, 89, 90, 110, 111). A strong positive correlation was observed between the two processes in a number of different microorganisms and in the synthesis of a variety of different proteins. Conversely inhibition of RNA synthesis inhibited protein synthesis. 5-OH uridine inhibited RNA synthesis in E. *coli*, it did not stop growth, but it inhibited synthesis of β-galactosidase. After depletion of pyrimidines in pyrimideneless mutants of yeast the synthesis of induced enzymes was greatly retarded; when purines and pyrimidines were added there was a prompt restoration of enzyme formation (71). Similar results were obtained by Pardee (106).

Gale and Folkes observed that the addition of purines and pyrimidines, RNA or DNA increased the rate of synthesis of protein including a number of enzymes in intact *Staph. aureus* (43, 110) and in the cell-free particles (45, 72). The effect on rates of synthesis varied with different amino acids. In general stimulation of synthesis of either RNA or protein stimulated the synthesis of the other.

From findings such as the foregoing, Spiegelman *et al.* (71) and Pardee (106) drew the suggestion that for the synthesis of protein new RNA must be synthesized concomitantly.

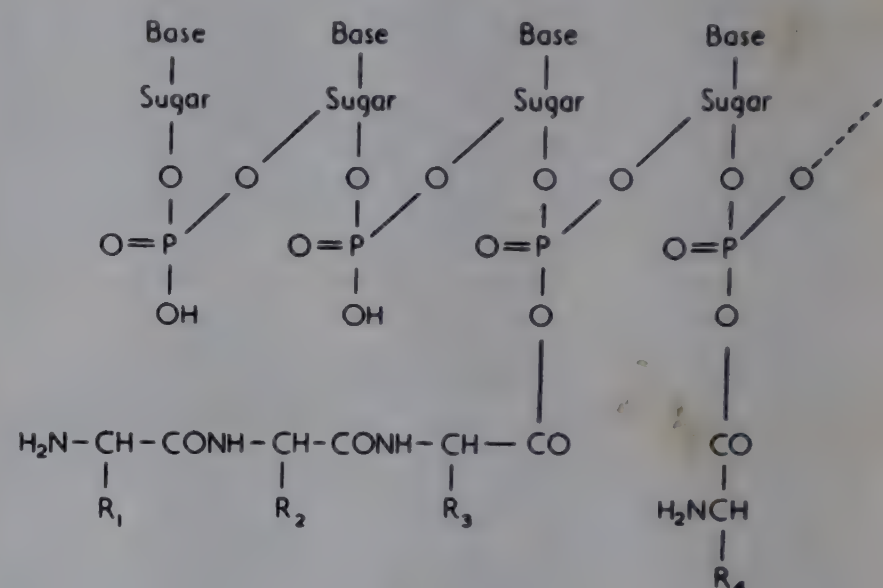
Stimulation of nucleic acid synthesis and protein synthesis do not always go together. Chloromycetin stimulates nucleic acid synthesis in *Staph. aureus* and inhibits protein synthesis (112). In the vitamin E deficient rat the turnover of both DNA and RNA in the intestine, liver and muscle is faster than normal without any significant difference in protein turnover (113).

In rabbit reticulocytes there is rapid protein synthesis at the same time as a rapid net decrease in RNA (114). These cells under optimal conditions synthesize hemoglobin at a rate of about 300 mg. in 4 hours per 100 ml. of cells. On the assumption that the molecular weight of RNA is about five times that of hemoglobin, if one molecule of RNA needs to be synthesized per molecule of hemoglobin synthesized, then 1 500 mg. of RNA would need to be synthesized in 4 hours, or twice the total RNA originally present, at a time when the RNA in these cells is rapidly decreasing. There is some synthesis of RNA; the rate could not be measured, but it appears to be only a very small fraction of 1 500 mg. in 4 hours (114).

An explanation that suggests itself is that only part of the RNA is active or native, the rest is inactive or denatured. Only native (active) RNA can serve as a template for protein synthesis. The RNA is continually being denatured and hence, for active protein synthesis, new RNA must continually be synthesized; but not necessarily on an equimolar basis. Hershey (115) in an analysis of the effects of bacteriophage on RNA and DNA metabolism in *E. coli* came to the conclusion that one must distinguish between the metabolically active RNA and the bulk of the RNA in the cell before infection.

### *Some consequences of the difference in structure of RNA and of protein*

The present view of the structure of RNA is that it is a rather shallow spiral. The pitch of the spiral is much less than that of proteins in their helical form. The consequence is that protein cannot take its normal form while attached to the template. There is less strain, not only to the protein and to our explanations if one assumes that on the template the carboxyl groups are attached and the amino groups are free, in other words there are no peptide bonds; that as soon as peptide bonds are formed that much of the peptide chain uncoils, and dangles, as it were, as in the following diagram :




For different reasons a picture of incomplete proteins dangling from the template has been proposed by Dalgliesh (116). In his picture many peptide chains at different stages of growth may be attached to the template at the same time.

The specificity of position of the amino acids on the template is determined by the topography of the space created by the purine and pyrimidine bases. Because the spiral is a shallow one the space for any one amino acid is made, not by the bases that immediately follow each other in the nucleotide chain, but by bases possibly as much as ten nucleotides distant.

Electron microscopic studies indicate that the RNA of the cytoplasm is a reticulum of vesicles and canaliculi (117, 118). Presumably the microsomes, which are the most active protein synthesizing material in the cell, are derived from this reticulum.

It is easier with a reticulated RNA template to envisage the formation of long peptide chains with disulfide cross links and reversals of the helix. At any rate there is direct evidence now for extended RNA which makes it easier to envisage it serving as a template.

*The relation of the level and the quality of nitrogen intake to the rate of protein synthesis*

Obviously no protein will be synthesized unless all the spaces on the template are filled by amino acids. The rate of protein synthesis will be dependent on the rate at which the template is filled. This rate will depend on the rate of activation of amino acids which in turn will depend on the rate of production of ATP and on the concentration of free amino acids. Table II shows the effect of amino acid composition on the rate of protein synthesis in rabbit reticulocytes. One may assume that in the cells of the blank there were some amino acids, and these were replenished at a slow rate by the breakdown of reticylocyte protein. The results show that a limitation of one amino acid limits the rate of the whole process. This limitation is not an absolute deficiency; it is a suboptimal concentration. It is significant that the concentration of amino acids in rabbit plasma was far from optimal; the rate could be more than doubled simply by enrichment with histidine, leucine, phenylalanine and valine (119).

The major effects of a protein meal on the nitrogen balance as on the specific dynamic action are within the first few hours after it is eaten, *i.e.* during the time that the amino acid level in the blood and tissues is increased. This has been observed with labeled amino acids (31, 75). Similarly it has been found in feeding experiments that an indispensable amino acid is ineffective for growth, for recovery from protein depletion, or for maintenance, unless it is fed or injected within a few hours of other necessary amino acids (120-129). So long as all the necessary free amino acids are in the tissues, which they always are, there is always some protein synthesis. A single labeled amino acid, whether dispensable or indispensable, is extensively incorporated into an animal's protein in a few minutes (31), whether the animal is in a normal nutritional state or fasting (130). It is a question of more or less synthesis. The critical parameter is the concentration of the amino acids and the completeness of the mixture. In an incomplete amino acid mixture the rate of protein synthesis will be slower the more incomplete it is and accordingly the greater will be the negative nitrogen balance. The difference in the biological quality of proteins or of amino acid mixtures resides in how high will be the concentration of all the needed amino acids in the tissues during the few hours after it is eaten. If a protein is short in one indispensable amino acid obviously more of it must be eaten to maintain such a level of protein synthesis that nitrogen balance (or growth) is maintained. The different amino acid mixtures in table II could be considered as different proteins. Tables III, IV and V show that intermediate effects can be obtained by adding graded amounts of any one of the limiting amino acids.

TABLE II.

*Effect of amino acid composition on the rate of protein synthesis* in vitro *in rabbit reticulocytes*

| Amino acid composition | Hours 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Complete | 447 | 450 | 489 | 475 |
| Complete without histidine | 94 | 87 | 94 | 96 |
| Complete without valine | 185 | 170 | 181 | 187 |
| Complete without phenylalanine | 176 | 183 | 181 | 200 |
| Complete without serine | 215 | 227 | 220 | 228 |
| Complete without lysine | 235 | 229 | 237 | 222 |
| Complete without tryptophane | 257 | 267 | 264 | 285 |
| Complete without tryosine | 262 | 280 | 295 | 297 |
| Complete without glutamine | 304 | 339 | 353 | 366 |

Results are expressed as % of blank (without added amino acids).

TABLE III.

*Effect of partial histidine deficiency on rate of protein synthesis* in vitro *in rabbit reticulocytes*

| Amount of histidine added with otherwise complete amino acid mixture | | Rate in successive hours 0-1 | 1-2 | 2-3 | 3-4 |
|---|---|---|---|---|---|
| (µg./ml.) | ($M \times 10^4$) | | | | |
| 0 | 0 | 23 | 18 | 27 | 23 |
| 9.25 | 0.575 | 81 | 30 | 33 | 24 |
| 18.5 | 1.15 | 104 | 77 | 39 | 25 |
| 27.75 | 1.72 | 100 | 98 | 93 | 47 |
| 37.0 | 2.30 | 100 | 98 | 88 | 80 |
| 46.25 | 2.87 | 100 | 97 | 98 | 102 |
| 74.0 | 5.75 | 100 | 100 | 100 | 100 |

Results are expressed as % of that with maximum histidine added.

TABLE IV.

*Effect of partial phenylalanine deficiency on rate of protein synthesis* in vitro *in rabbit reticulocytes*

| Amount of phenylalanine added with otherwise complete amino acid mixture | | Rate in successive hours 0-1 | 1-2 | 2-3 | 3-4 |
|---|---|---|---|---|---|
| (µg./ml.) | ($M \times 10^4$) | | | | |
| 0 | 0 | 35 | 37 | 39 | 38 |
| 6.5 | 0.39 | 88 | 66 | 62 | 61 |
| 13.0 | 0.78 | 90 | 90 | 87 | 80 |
| 19.5 | 1.18 | 92 | 93 | 105 | 98 |
| 26.0 | 1.57 | 93 | 92 | 100 | 102 |
| 32.5 | 1.96 | 94 | 93 | 102 | 101 |
| 65.0 | 3.93 | 100 | 100 | 100 | 100 |

Results are expressed as % of that with maximum phenylalanine added.

TABLE V.

*Effect of partial valine deficiency on rate of protein synthesis* in vitro *in rabbit reticulocytes*

| Amount of valine added with otherwise complete amino acid mixture | | Rate in successive hours | | | |
|---|---|---|---|---|---|
| | | 0-1 | 1-2 | 2-3 | 3-4 |
| (µg./ml.) | (M × $10^4$) | | | | |
| 0 | 0 | — | 29 | — | 32 |
| 9.25 | 0.78 | 81 | 61 | 50 | 53 |
| 18.5 | 1.57 | 98 | 85 | 71 | 73 |
| 27.75 | 2.36 | 100 | 97 | 84 | 86 |
| 37.0 | 3.15 | 95 | 99 | 92 | 98 |
| 46.25 | 3.94 | 95 | 99 | 94 | 97 |
| 92.5 | 7.89 | 100 | 100 | 100 | 100 |

Results are expressed as % of that with maximum valine added.

### *Slowness of protein synthesis*

Protein synthesis is slow as compared with other enzymatic reactions. For purposes of comparison we may consider the whole process as if it were mediated by a single enzyme. If we assume that the molecular weights of the protein and of the hypothetical enzyme are the same, then the turnover number of the enzyme is

$$\frac{\text{Weight of protein synthesized per minute}}{\text{Weight of enzyme}}$$

In reticulocytes the maximum rate of hemoglobin synthesis we have observed was 6.4 mg. per gram of total protein per hour. If we assume that the enzyme was of the order of 1 % of the total protein, then the rate was 640 mg. per gram of enzyme per hour or 10.6 mg. per gram of enzyme per minute, or a turnover number of approximately $10^{-2}$. If we take the turnover number as the number of amino acid residues built into protein, with approximately 580 amino acid residues per mole of hemoglobin (131), the turnover number per residue is 5.8. The turnover number of ordinary enzyme reactions is from 100 to 3 000 000. Even in bacteria during the logarithmic phase of their growth, when protein synthesis is 1000 times faster than the fastest rates in mammalian tissues, the turnover number per amino acid residue is of the order of 500 which is in the slow range of ordinary enzyme rates.

### *Inhibition on the template*

*A priori* it is to be expected that an inhibitory amino acid analogue would block its natural counterpart on the template and thereby block the utilization of all the other unrelated amino acids for protein synthesis. Halvorson and Speigelman (70) demonstrated this effect in yeast with inhibitory analogues of leucine, methionine and phenylalanine; only the natural counterpart could relieve the inhibition.

In at least one case the inhibitory analogue is incorporated, *i.e.* an abnormal protein is formed. This is the case with ethione in the rat (132). As was to be expected it was incorporated more slowly and its « half-life » in the tissue proteins was shorter than that of its natural counterpart, methionine, and it slowed down the incorporation of other amino acids into protein.

Peptides accumulate in microorganisms when protein synthesis is deranged as in *Staph. aureus* with glucose and an incomplete amino acid mixture (72, 133, 134), or in the presence of penicillin (135, 136). Among these are the peptides Park has isolated and found to be attached to uridine nucleotides (135). These peptides may be incompletely formed protein which come off the template as a result of the derangement by an incomplete amino acid mixture or by the antibiotics. Chloromycetin and aureomycin have a small if any inhibiting effect on the formation of these peptides (133). The findings as a whole point to interference on the template as the locus of action of chloromycetin and penicillin. Aureomycin which, at 400 µg./ml. uncouples oxidative phosphorylation (137) would at this concentration inhibit the activation process; the data of Gale and Paine (42) suggest that this is the case; they found that peptide formation is inhibited by a lower concentration of aureomycin than of chloromycetin. But both antibiotics inhibit protein synthesis at much lower concentrations, *e.g.* 20 µg./ml. (72, 132, 137). At this concentration their inhibitory action is at the template. One can hardly expect to find such peptides except where protein synthesis is very rapid, as in microorganisms.

### *Amino acid exchange in proteins*

Operationally amino acid exchange means one of two findings : unequal labeling of a given amino acid at different loci in the protein molecule, or incorporation of an amino acid into a protein under conditions where synthesis of protein *de novo* from amino acids is excluded.

We have referred above to the unequal labeling observed by Anfinsen and his coworkers. The inequality of labeling is greatest at the beginning of an experiment, it grows progressively less with time, and this variation in the inequality of the labeling is not due to changes in the specific activity of the free amino acid.

An explanation which brings these observations into line with a number of others is as follows. Protein synthesis is a slow reaction. For a finite time certain loci on the template are unoccupied and the protein cannot be released to the medium until all the loci are occupied. During this time the amino acids bound to the template but not to each other can exchange with other activated amino acid coming from the free amino acid pool. At the beginning of an experiment the empty spaces on the template will be filled by labeled amino acid, and also the labeled amino acid may exchange with unlabeled amino acid on the template, at different rates at different loci. Both processes would give unequal labeling when, after a time, the template is fully occupied, the protein is released from the template. From then on the same amino acid will be equally labeled at all loci in the protein molecule.

The second kind of amino acid exchange, incorporation of an amino acid into a protein under conditions where synthesis of protein *de novo* from amino acids is excluded,

has been observed experimentally. We exclude here the special case of lysine incorporation into a guinea pig liver protein (1, 139). We refer to experiments such as those described by Gale and Folkes (44, 45, 72) where washed *Staph. aureus* supplied with a single amino acid and a source of energy, incorporated the amino acid into its protein, and there was no increase in cellular protein. The incorporation was inhibited by inhibitors which prevent ATP formation, *i.e.* amino acid activation. That an actual transfer occurs was proved by beginning with labeled protein in the bacteria and finding labeled amino acid, after the exchange, free in the medium.

This kind of exchange appears to be the most reasonable explanation for the observations of Rabinovitz *et al.* (140) that leucine, lysine and valine were incorporated into ascites carcinoma cells in the presence of a concentration of a phenylalanine analogue that blocked completely incorporation of phenylalanine.

In the tissue culture experiments of Francis and Winnick (141) the findings on the effects of amino acid analogue inhibitors suggest (they are not proof) that the incorporation of free amino acids can be inhibited while proteins (or protein fragments) remain available for limited protein synthesis or for turnover processes.

It has been found in rats whose proteins were labeled with either glycine or alanine that the rate of disappearance of the labeled amino acid from the animal's proteins was greater when glycine or alanine respectively was continuously infused. Alanine was « washed out » best by alanine, glycine by glycine (142).

This second mode of amino acid exchange is readily accounted for if one postulates that preformed protein can be, and is, reimposed on the template. If the terminal carboxyl group is to be attached it would require activation, *i.e.* utilization of ATP. A part of the protein might be attached at the locus of an internal peptide bond *via* cleavage of the protein, and with the energy of the peptide bond conserved by attachment of the newly formed carboxyl group to the enzyme and thence to the template. To the extent that the protein or peptide is attached to the template all its peptide bonds are broken, the rest dangles from the template. Exchange would occur only in those parts that are attached to the template. Energy is required for the exchange because free amino acids of the pool must be activated before they can exchange.

In general it would be expected from considerations of the geometry and probability that re-imposition of a protein or peptide on the template would be a much slower process than protein synthesis *de novo* from amino acids and that there is a competition for the template between activated amino acids and preformed protein. The rate of turnover of a preformed protein would be governed by the amount of enzyme available for attaching it to the template and the competition from new protein synthesis from amino acids.

The process envisaged is :

$$\text{Protein} \rightleftarrows \text{(Template) A-A} \rightleftarrows \text{Activated A-A} \rightleftarrows \text{Free A-A.}$$

It is by a scheme such as this that nitrogen balance in animals is maintained. An increase in free amino acids increases synthesis of protein, increase of any one kind of protein (so long as it is not, like hemoglobin, excluded from metabolism) increases the rate of breakdown of that protein. Hence the close correlation in different tissues between overall turnover as measured over periods of days by $^{15}N$ labeling and rates of incorporation *in vitro* or *in vivo* measured in an hour or two (139).

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## The mechanism of protein biosynthesis (*)

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(*Received* 6 *July* 1955)

In our laboratory we have been interested for many years in antibody formation which is a special type of protein biosynthesis. In 1930, together with Breinl, I proposed a hypothesis according to which the antigen interferes with the formation of normal globulins from amino acids in such a fashion that a globulin molecule is formed which is complementarily adapted to the determinant group of the antigen (1). Similar hypotheses were shortly thereafter published by Mudd (2) and Alexander (3). We thought at that time that the antigen might affect the sequence of amino acids in the peptide chain of the antibody molecule. Later Pauling (4) suggested that the antigen may interfere with the folding of the peptide chain only. This view is supported by experiments in our laboratory. We prepared in these experiments antibodies against two azoproteins, one of them prepared by coupling beef serum gamma-globulin with diazotized anthranilic acid, the other by coupling the same protein with aminophenyltrimethylammonium ions. One of these antigens has an acidic group, whereas the other has a strongly basic quaternary ammonium ion as determinant group. We purified the two antibodies by dissociation of the antigen-antibody precipitates (5). The amino acid analyses did not reveal any significant difference between these two antibodies and normal rabbit serum gamma globulin (6). Recently, Smith (7) and Porter (8) have determined the N-terminal amino acids of rabbit antibodies and the adjacent 4 amino acids. They found in all cases the same sequence. It is quite possible, therefore, that all rabbit antibodies have the same amino acid composition and amino acid sequence, and that they differ from each other only by the mode of folding of their peptide chains.

Since antibody formation is nothing but a special type of protein biosynthesis, I tried later to extend our theory so that it comprised protein formation in general (9). It became clear at that time that we needed something to explain the specific sequence of the amino acids in the peptide chain and I invoked the existence of an organizer or template which would align the amino acids in the unique sequence which is a characteristic property of the species-specific peptide chain. It became necessary to postulate at least two steps in protein formation, one of them consisting of the formation of the peptide chain from amino acids, the second of the folding or coiling of the peptide chain to give a three-dimensional, globular molecule. In order to explain the specific sequence of amino acids in the peptide chain, I suggested that the template is formed by the same protein molecule as an expanded monomolecular film. I assigned to the nucleic acid mainly the function of keeping the protein template in the expanded state.

In the five years since I proposed this mechanism, it has undergone some changes in the hands of other biochemists, although the principal ideas remained unchanged. These are the ideas (*a*) that the specific sequence of amino acids is due to a template, whereas

(*) Supported by the U. S. Public Health Service, the National Science Foundation, the Atomic Energy Commission and the Office of Naval Research.

the action of the enzymes which catalyze formation of the peptide bonds is more or less nonspecific, and (*b*) that the alignment of amino acids is an initial phase, with subsequent condensation and folding as a final phase. The most important change in this picture involves the role assigned by Dounce (10), and now by Borsook (11) to the nucleic acid. I am somewhat reluctant to accept this view because the species-specificity of proteins seems to be an inherited property, transmitted from mother cell to daughter cell; such characteristics in general are attributed to the action of the genes which contain desoxyribonucleic acid but no ribonucleic acid.

While the specific amino acid pattern of the peptide chain is an inalterable, inherited characteristic, acquired immunity is never transmitted from generation to generation. We assume, therefore, that the folding of the peptide chain, which results in antibody formation, takes place in the cytoplasm, not in the nucleus. Investigations with radioactive antigens have shown that some of these antigens are concentrated in the cytoplasm (12). This supports our view that the folding of the peptide chains is a cytoplasmic process. However, we do not know whether the first phase, the formation of the peptide chain from amino acids occurs in the nucleus or in the cytoplasm.

Our views on protein formation from amino acids without the formation of peptides as intermediates, has been criticized by Synge (13) who considers a multimolecular reaction as extremely improbable. It must be emphasized, therefore, that formation of proteins from amino acids does not involve multimolecular reactions. We believe that one amino acid after the other is adsorbed to or combines with the template molecule; later, peptide bonds may be formed between adjacent amino acids in a zipper-like fashion. Each of these reactions is of the transfer reaction type, hence a bimolecular reaction.

Although nobody has ever seriously proposed that a protein macromolecule such as hemocyanin is formed by the multimolecular instantaneous combination of thousands of amino acids, it would be interesting to know how fast protein molecules are formed. Immunochemical investigations allow us to make such an estimate. We know that about 1 microgram of a powerful antigen such as diphtheria toxoid is able to give rise to the formation of approximately 1 gram of antitoxin in the course of three weeks (14). Assuming approximately identical molecular weights for toxoid and antitoxin this gives 1 million of antitoxin molecules per toxoid molecule in three weeks. If we assume that each toxoid molecule cannot act upon more than one antitoxin molecule at a time, this corresponds to a period of two seconds for the formation of an antitoxin molecule. Since it is improbable that all of the injected toxoid molecules are involved in antitoxin synthesis at the same time, the time of 2 seconds per protein molecule is a maximum time. The true time required for the biosynthesis of a protein molecule may be much shorter.

Finally I want to report briefly on recent experiments on protein formation *in vitro*. We have investigated the old and well-known phenomenon of plastein formation using radioactive amino acid derivatives. It was shown first by Danilewski in 1886 that proteinfree peptic digests when they are neutralized, concentrated, and incubated with pepsin form an insoluble protein. This was called plastein by another Russian author, Sawjalow. The phenomenon was later investigated by Borsook (15), Virtanen (16), Tauber (17), and others. It became clear by these investigations that pure crystalline chymotrypsin catalyzes the formation of an insoluble protein in neutral peptic digests. We have examined this reaction using radioactive phenylalanine, which is a typical substrate for chymotrypsin. We found no incorporation of phenylalanine into the plastein formed. However, we find definite incorporation when we add phenylalanine ethyl ester (18). Neither glycine nor glycine ester is incorporated into the plastein formed under the same conditions. It is clear from our experiments that plastein formation essentially consists of transpeptidation. The phenylalanyl residue is transferred from ethanol to the plastein molecule, whereas free phenylalanine cannot be utilized. The ease with which this reaction takes place *in vitro* under physiological conditions suggests that similar transfer reactions may also take place in the organism. They would explain the variety of protein formed by means of a single template. The primary product of protein synthesis, after its removal from the template may exchange by such transfer reactions parts of its peptide chain and may, thereby, give rise to the formation of new types of proteins. We do not yet know whether this type of reactions plays a major role in the biosynthesis of proteins. It may be responsible for the heterogeneity of many of the natural proteins, and for small differences in their structure.

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# The structure of polypeptides and proteins (*)

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(*Received* 4 *April* 1955)

## SYNTHETIC POLYPEPTIDES

### *Introduction*

As a result of numerous observations of infra-red spectra and of X-ray diffraction photographs, a good deal is now known about the structure of synthetic polypeptides of high molecular weight containing identical residues in which the side chains are inert. Less is known about polypeptides with identical acidic or basic residues, though the few which have been examined appear to have marked structural resemblances to the inert polypeptides; practically nothing is known concerning the structure of synthetic polypeptides in which there are mixtures of active and inert residues.

The important result which has emerged is that the synthetic polypeptides with inert side chains may exist in either of two stable configurations. The arrangement of the atoms in the polypeptide backbone in these two forms is to a large extent independent of the size of the side chains or R groups which characterise the polypeptide. In one of these forms the polypeptide chains are extended or nearly so (β form) and the individual chains are formed into flat sheets in which NH—O hydrogen bonds connect the peptide groups of neighbouring chains. In the folded (α) form all the potential hydrogen bonds are satisfied within one molecular chain, which is coiled into a helix in which consecutive turns are linked by NH—O hydrogen bonds, and these helices pack in a more or less hexagonal arrangement in crystallites. The broad classification into these two forms, α and β was foreshadowed by Astbury and his coworkers' investigations on keratin (1, 2) and by considerations put forward by Taylor (3) and Huggins (4) before the synthesis of polypeptides of high molecular weight had allowed experimental work on their properties to be carried out. Following the discovery of a folded configuration in poly-γ-methyl-L-glutamate by the use of polarised infra-red radiation (Ambrose and Hanby, 5), X-ray diffraction photographs of oriented films of this and other polypeptides were published (Bamford, Hanby and Happey, 6, 7), and it was found that X-ray powder photographs as well as infra-red spectra showed that polypeptides could be produced in two distinct chain configurations. The particular type of helix (the α-helix) referred to above was described in 1951 by Pauling, Corey and Branson (8), and it was pointed out by Pauling and Corey (9) that the X-ray diffraction photograph of poly-γ-methyl-L-glutamate could be interpreted as an α-helix pattern. The correctness of this interpretation was strengthened when a strong reflection corresponding to a spacing of 1.5 Å was found in a diffraction photograph of α poly-γ-benzyl-L-glutamate (Perutz, 10).

Further confirmation was provided when Cochran and Crick (11) calculated the main features of the intensity distribution on an X-ray diffraction photograph of an oriented polypeptide fibre in which the molecules were coiled as α-helices, and Bamford, Brown, Elliott, Hanby and Trotter (12) obtained greatly improved X-ray photographs of poly-γ-methyl-L-glutamate which showed just these features.

Further references and much information on the earlier development of the subject may be found in the report on a discussion on the structure of proteins (13).

### *Evidence for the Pauling-Corey α-helix in synthetic polypeptides*

*X-ray diffraction photographs of poly-L-alanine.* — The first evidence for the existence of α helices was based on X-ray diffraction photographs of poly-γ-methyl-L-glutamate and poly-γ-benzyl-L-glutamate, both of which have large and flexible side chains attached to the α-carbon atom. To account for the reflections observed in their X-ray diffraction photographs quantitatively is an impossible task at present, since it is not even certain that the side chain arrangement is regular.

With poly-alanine the situation is different, because if the coordinates of the atoms in the spiral are given, the β-carbon atom of the methyl group has only two possible positions (referred to as $\beta C_1$ and $\beta C_2$). In a right-handed helix, these are the β-carbon atoms in poly-L and poly-D-alanine, respectively (14). In a left-handed helix, these positions are interchanged. It

(*) Because of the need to keep this report within the limits prescribed, it is not practicable to deal adequately with every aspect of the physical structure of polypeptides and proteins. To have touched upon every topic would have been to deal satisfactorily with nothing: the reporter has, accordingly, omitted reference to much interesting work in order that those topics which have been included might have adequate discussion.

should therefore be possible to examine the limited number of possibilities and to decide whether the α-helix coordinates given by Pauling and Corey (15) are compatible with the observed intensities in the X-ray diffraction photograph.

It has been found possible to obtain very good X-ray photographs from well-oriented poly-L-alanine fibres (Bamford, *et al.*, 16) and some work has been done on the comparison of calculated and observed intensities. The equatorial reflections have been found to fit quite well, but on the strong 5.4 Å layer line neither $\beta C_1$ nor $\beta C_2$ positions lead to predicted intensities which agree with those observed.

*The optical transform.* — An important development of an optical aid in crystallographic investigations, originally due to Bragg (17) has recently been made by Lipson and his co-workers (see, for instance, Hanson, Lipson and Taylor, 18). This is already being used by several groups working on the structure of fibrous proteins and polypeptides. In brief, one aspect or view of a proposed structure is examined by projecting the atomic positions on a chosen plane. By means of a pantograph or by photography, the atomic positions on this plane are represented by transparent holes in an opaque screen, which is then placed in front of a telescope lens and illuminated by parallel light. The diffraction pattern formed at the telescope focus is known as the 'optical transform' of the assumed atomic arrangement; it enables the intensity distribution in an X-ray diffraction photograph of a molecule which has the same arrangement of atoms to be predicted without the lengthy calculations necessary by the usual methods. Preliminary results on the optical transform of the α-helix for poly-L-alanine (Courtaulds Laboratories, unpublished) show that, as mentioned above, neither $\beta C_1$ nor $\beta C_2$ appear to allow a match between observed and calculated intensities to be made.

Both the calculations of Cochran and Crick and the observations on the optical transform leave no room for doubt that the structure of α poly-L-alanine is a helical arrangement of peptide residues in which each residue is related to the next by a screw with an axial translation of *ca.* 1.50 Å and a rotation of *ca.* 100° C. What the X-ray evidence does not yet show is the precise arrangement of atoms in each residue, and on this evidence alone it is not yet possible to decide between the α-helix of Pauling and Corey and another helix of eleven-atom rings (Bamford *et al.*, 19; Huggins, 20), though no doubt further work will settle this. The view that the α-helix is essentially correct, now widely held, is based on an extrapolation from the structural characteristics of the amide group, particularly the planar nature of this group, in simple compounds. A good exposition of the subject has been given by Crick (21).

*Infra-red dichroism.* — In α polypeptides, the optical density of the C = 0 stretching band in a well-oriented film, like that of the NH stretching band, is greater when measured with the *E* vector of the incident radiation parallel to the fibre axis than when it is perpendicular (Ambrose and Elliott, 22). However, this effect (dichroism) is much more marked in the NH band than in the C = 0 band. In the α-helix the NH and C = 0 bonds are both inclined at approximately 12° C. to the helix axis, and this should lead to very high dichroism in all infra-red bands in which the molecular motion involved results only in stretching one or the other of these bonds.

It is now known that the molecular motion which is concerned with the C = 0 band is complex, and that the observed dichroism does not at once lead to a knowledge of the direction which the C = 0 bond makes with the molecular axis (Bamford *et al.*, 23; Fraser and Price, 24; Elliott, 25; Abbott and Elliott, 26). The phenomenon of resonance in the amide group is chiefly responsible for the effects observed, which however require further investigation before they can be accounted for quantitatively.

### *Recent developments in spectroscopic observations on polypeptides*

Early observations on a small number of polypeptides showed that, whereas the frequency of the absorption band associated with stretching of the NH bond remained at *ca.* 3300 cm.$^{-1}$ in both α and β polypeptides, the other two strong amide bands (C = 0 stretching mode and a band of complex origin chiefly associated with NH in-plane deformation) had frequencies which were characteristic of α and β forms (22). These results have been confirmed and amplified in polypeptides where the effect of side chain size and character has been examined. The C = 0 band, especially, has characteristic frequencies in α and β forms which, in simple polypeptides, are independent of side chain character. In optically active α polypeptides, this band occurs in the range 1652-1657 cm.$^{-1}$, and in *meso* forms the range is somewhat higher, namely 1661-1665 cm.$^{-1}$. In β forms, for which the observations are less complete, the range is 1628-1635 cm.$^{-1}$ (Elliott, 27).

Development of techniques for orienting films and fibres of poly-L-alanine in both α and β forms have allowed observations of infra-red spectra which confirm and extend earlier conclusions, especially in the region of overtone and combination bands (Elliott, Hanby and Malcolm, 28). This region is important because it allows observations on some proteins which would otherwise be difficult or impossible.

Observation of the NH out-of-plane deformation mode at *ca.* 700 cm.$^{-1}$ promises also to be of importance in the interpretation of polypeptide and protein spectra (Kessler and Sutherland, 29).

### *The extended chain (β) configuration in synthetic polypeptides*

*The repeat distance along the chain axis* (c *repeat*). — Measurements of bond lengths and angles in amide groups in simple substances lead to a predicted repeat distance in a fully extended polypeptide chain of 7.23 Å (Corey and Pauling, 30). It has generally been assumed that polyglycine would have this configuration, but that in other polypeptides, steric effects would lead to buckling and contraction (Huggins, 4; Pauling and Corey, 31). Recently some experimental values for the dimensions

of the $c$ axis repeat of β polypeptides have been made (Bamford *et al.*, 32), and these are given in table I. Since polyglycine was not examined in an oriented form, the $c$ axis determination is not so certain as in the other polypeptides, but good reasons are given for believing that it lies within the range quoted. It seems likely that polyglycine chains are not fully extended, though it is possible that the predicted length 7.23 Å needs revision. On the whole, the $c$ axis seems to shorten with increase in size of the side chains, and there is no evidence to suggest that a precisely specific β-configuration exists which is independent of side chains. Pauling and Corey (31) have published coordinates for several specific chain configurations, all nearly extended. These are based on energy considerations (linear hydrogen bond, staggered arrangement of bonds, etc.). In general, they find that the length of the $c$ axis repeat depends on whether all chains connected by hydrogen bonds have the sequence CO-NH-CHR in the same direction ('parallel' chains) or whether this sequence is in opposite directions in consecutive chains ('antiparallel' chains). They predict a chain axis repeat of 7.00 Å for an antiparallel arrangement, which is close to the value for polyglycine and for silk fibroin (table 1).

TABLE I.

| Material | $c$-Axis (Å) | Mean residue weight |
|---|---|---|
| Polyglycine (β-form) | 6.97-7.05 | 57 |
| Poly-L-alanine (β-form) | 6.88 | 81 |
| Poly-γ-methyl-L-glutamate (β-form) | 6.83 | 143 |
| Silk (*Bombyx mori*) | 6.94 | 77 |
| Silk (tussah) | 6.92 | |
| Pig-bristle (stretched) | 6.62 | 107-116 |
| Swan quill | 6.2 ? | |

*Packing of β-chains.* — The general arrangement of chains in β structures is apparently similar to that found in nylon by Bunn and Garner (33) and is shown in fig. 1.


FIG. 1.

The chains are connected by hydrogen bonds and form sheets, between which only van der Waals forces operate. The angle γ apparently varies from about 66° C. in polyglycine to nearly 90° C. in β keratin (see Astbury, 34) and may depend largely on side chain size.

Recently Brown and Trotter (35) have considered the chain packing in β poly-L-alanine, and find that there are more reflections in the diffraction photograph than can be accounted for by the unit cell of fig. 1, in which there is one chain per cell. This means that, in a crystallite, the chains are not all 'parallel.' Whether the actual arrangement is regular, or a random arrangement of parallel and anti-parallel chains, is not known.

### *Other folded polypeptide chain configurations*

*Theoretical considerations.* — Following the methods of examining possible ways of folding polypeptide chains which Pauling and Corey used in predicting the α-helix, Low and Baybutt (36) and Donohue (37) have considered the stability of other folds than the α- and γ-helices. They adopted the assumptions made by Pauling and Corey (planar amide group, nearly linear NH—O hydrogen bond, equivalence of residues and bond distances and angles as found in simple compounds) but allowed slight departures from the rigorous assumptions. In all, four additional helices are found to merit consideration, though none is, as an individual chain, as stable as the α-helix. It seems possible that one or more of these helices might be found if side groups affected the energy in a system of packed chains.

*A new configuration in polyglycine?* — No evidence of these folds has been found so far, but there is definite experimental evidence of a hitherto unrecognised configuration in polyglycine (polyglycine II) (Elliott and Malcolm, 38). Meyer and Go (39) showed that two different X-ray powder photographs could be obtained from polyglycine, according to the method of preparation used. One of these is certainly the extended chain form; the powder photograph shows a strong ring corresponding to a spacing of 1.16 Å, a spacing which is characteristic of β forms (Bamford *et al.*, 32) and a rolled specimen shows the characteristic dichroism of these forms. The other form, obtained by precipitation from solution, is characterised by an infra-red spectrum in which the $C = 0$ band is at 1648 cm.$^{-1}$; the whole of the infra-red spectrum is different from that of β polyglycine. The X-ray powder photograph of polyglycine II is also highly characteristic; no 1.16 Å reflection is observed and the strongest ring corresponds to a spacing of 4.15 Å. Considerations of density show that this powder photograph does not arise from α-helices. At present it is not known whether polyglycine II has inter- or intra-chain hydrogen bonds.

### *Polypeptides in solution*

*Evidence of α-helix in solution.* — Work on the physical structure of polypeptides in solution has been confined to those with inert side chains. In inert solutions such as benzene, and carbon tetrachloride, only α polypeptides are soluble (Bamford, Hanby and Happey, 6, 7) and there is now evidence that the α-helix form is retained in solution, at least when the molecular weight is high. Thus, the wave number of the $C = 0$ stretching band is little different in solid films and in solution (Elliott, 27).

Evidence of a different kind has come from light-scattering experiments, which show that molecules of poly-γ-benzyl-L-glutamate in chloroform behave as if they were rods of length $M \times 1.5$ Å where $M$ is the average number of residues per molecule (Doty, Holtzer, Bradbury and Blout, 40) and this is the relation expected in an α-helix where the translation per residue is 1.5 Å. The diameter of the rods is 16 Å, a reasonable value for a solvated helix.

*Paracrystalline forms.* — Poly-γ-benzyl-L-glutamate in solution in chloroform has been known to show a phase separation at a suitable concentration; one of these phases consists of, or contains spherulites, and possesses marked self-orienting properties (Elliott and Ambrose, 41). This interesting polymer has been examined more extensively recently by Robinson (42), particularly in solution in dioxane or methylene chloride. As might be expected, it is found that the concentration at which phase separation begins is dependent on molecular weight. The spherulites in the birefringent phase show structure which at first sight suggests a number of concentric shells; however, since the image of the spherulite on closer examination shows a continuous spiral instead of closed shells, it is not certain how these observations are to be interpreted. The birefringent phase shows a strong tendency to form layers, in which the refractive index changes periodically. When flat layers are formed (as happens near the surfaces of a rectangular glass cell) it is found that there is very strong optical rotation produced in a plane polarised light beam incident normally on the layers. The degree of rotation is reminiscent of such phenomena in cholesteric liquid crystals, and suggests spiral structure, possibly on a macro scale.

## FIBROUS PROTEINS

### *Introduction*

The first contribution made by X-ray crystallography to the subject of protein structure came from an examination of fibrous proteins. From the crystallographic point of view, these substances are very 'poor relations' indeed of the crystalline globular proteins, but it is a singular fact that even to-day they contribute more positive knowledge of physical structure than do the much more regular structures. The reason lies undoubtedly in the fact that fibrous proteins contain chains sufficiently long for the basic structure of the chains to show characteristic diffraction patterns, whereas in globular proteins the chains can only be straight for comparatively short distances.

### *Structure of α keratin*

The X-ray diffraction photograph of porcupine quill contains many sharp reflections (MacArthur, 43), whereas that of mammalian hair has fewer, most of the diffracted radiation going into diffuse rings. Both these forms of α-keratin have their strongest reflections in common, however; these are a very strong reflection corresponding to a spacing of 9.8 Å on the equator (composite in the case of porcupine quill), and meridian reflections corresponding to 5.15 Å and 1.49 Å. It is commonly assumed that the structure is essentially the same in both materials, the differences being caused by a lower degree of order or crystallinity in hair. This is supported by the fact that both forms of α-keratin show similar dichroism in the main infra-red absorption bands (Ambrose and Elliott, 44); this dichroism is qualitatively similar to that observed in α polypeptides (22) though much lower than, for example, in well-oriented poly-γ-benzyl-L-glutamate.

The general similarity in the distribution of diffracted intensity over the X-ray photograph, and especially the occurrence of a 1.49-1.50 Å meridian reflection in the case both of α keratin and α polypeptides makes it very likely that a similar helical structure occurs in both materials. However, there is a difficulty in connection with the occurrence of a strong 5.15 Å meridian reflection in α keratin, for an α-helix, although it produces a strong layer line corresponding to about 5.4 Å, produces no reflection on the meridian except the 1.5 Å reflection (and orders at 0.75 Å etc.). In addition, if it is assumed that the 9.8 Å reflection is the $10\bar{1}0$ reflection of an approximately hexagonal cell, the calculated density is about 1.1 g./cm³, much lower than the measured value 1.32 g./cm³. Actually, the unit cell is known to be larger than is assumed in this calculation, for Astbury has observed an equatorial reflection of 27 Å (1, 2).

The possibility that crystallites containing folded helical chains could give rise to meridional 1.49 and 5.15 Å reflections if the α-helix axis were not straight has been put forward simultaneously by Crick (45, 46) and by Pauling and Corey (47). These authors have considered a 'coiled coil' structure, in which the axis of an α-helix is itself helical, though with a very long pitch and small diameter. Such an arrangement does allow both meridian reflections to appear.

Crick considers that packing considerations alone would produce such a structure, and that the coiled coils would pack like the strands in a rope, with the side chains of one helix fitting into spaces between the side chains of another helix. However, it must be noted that the structure of α poly-L-alanine, where such effects might be expected, does not show them.

Pauling and Corey suggest that, in α-keratin, the nature of the side chains is responsible for the secondary helix. They suggest an arrangement of seven-strand cables (in which each strand is a coiled coil) packed hexagonally at the corners of a unit cell of side 32.4 Å, with two single interstitial coiled coils of different type (and hence of different amino-acid composition) fitting in between the larger structures. This involves the assumption of three kinds of molecular chain, since the central strand of the seven-strand cable differs from the others. The distribution of intensity on the equator is roughly accounted for by this arrangement when calculations are made which, however, as the authors point out, only take account of half the scattering matter present. Whether the agreement will be improved on extending the calculations remains to be seen. The calculated density is somewhat low (1.17-1.30 g./cm³ depending on the value assumed for the mean residue weight).

The coiled-coil arrangement does not introduce a sufficient departure of the α-helix axis from the fibre axis to account for the very low dichroism of the NH

stretching mode in the spectrum of porcupine quill (Ambrose, Elliott and Temple, 48), and if the crystal structure implied in the idea of coiled coils is substantially correct, other factors must operate to produce the observed low dichroism; some have been suggested by Parker (49) and it is altogether likely that the chief one is the presence of amorphous material. Mere dis-orientation of crystallites would produce a ring corresponding to 9.8 Å, and of this there is no sign in MacArthur's photograph of porcupine quill. It appears to the reporter that, although there may be coiled coils or similar structures in α keratin, there may also be a good deal of material whose structure is quite unknown.

### *Muscle proteins*

The protein of muscle was classed by Astbury along with keratin as a folded, α polypeptide structure, and the 1.5 Å meridian reflection has been found in muscle (Huxley and Perutz, 50). Pauling and Corey suggested that muscle contraction involved a change from extended polypeptide chains to an α-helix (51). It was, however, found by Astbury and Dickinson that the α diffraction pattern persisted in the X-ray photograph of extended muscle (52).

Malcolm has recently examined the infra-red spectrum of live as well as of dried muscle in the overtone region (53) and has found no evidence of β polypeptide chains in extended muscle. This rules out the possibility, hitherto not excluded, that an α — β conversion takes place in amorphous regions of muscle when it is extended. He found the α type of dichroism in all states examined; as in α keratin, the dichroic ratio was very low, in this case 1.2:1. The band which he examined is highly dichroic in oriented α poly-L-alanine (dichroic ratio 7:1) where there is good evidence for α-helices. The low dichroism in muscle suggests that the oriented α-helices which produce the characteristic X-ray pattern only account for about 15 % of the protein in muscle, and that the remaining 85 % is not dichroic. Morales and Cecchini (54) have likewise found low dichroism in single muscle fibres in the region of fundamental bands. It would appear that the structure of the greater part of the protein in muscle is unknown.

### *The proteins of silk*

*Fibroin.* — Early work on fibroin, which is summarised in two recent papers (55, 56) has made it highly probable that the crystal structure consists of extended polypeptide chains hydrogen-bonded into sheets, and that the pseudo-unit is similar to the monoclinic cell suggested for polyglycine and β-poly-L-alanine (above). Warwicker (55) has put forward a structure for *Bombyx mori* silk in which each hydrogen-bonded sheet is packed with respect to neighbouring sheets in an orthogonal manner, which would suggest an essentially different kind of packing. This structure has been severely criticised by Marsh, Corey and Pauling (56, 57) who have pointed out major discrepancies between the observed X-ray diffraction photograph and intensities in the diffraction diagram calculated from Warwicker's parameters.

Marsh, Corey and Pauling have described a pseudo unit cell with $a = 9.40$ Å, $b = 6.97$ Å (fibre axis), $c = 9.20$ Å and $\beta = 90°$ C. which, although orthogonal, is monoclinic in symmetry, and have given parameters for the atoms in the polypeptide chain and for the β carbon atom. There are four chains in the pseudo unit cell, and the packing is similar to that suggested for polyglycine and for β-poly-L-alanine in that the chains in one hydrogen-bonded sheet are staggered with respect to those in neighbouring sheets; this is almost certainly demanded by packing considerations in polypeptides with small side chains. The agreement between observed and calculated intensities in the X-ray diffraction pattern is fairly good, considering the difficulties inherent in the problem; it is, however pointed out by the authors that a larger cell is required to explain the whole diffraction pattern.

The implications of the proposed structure are interesting. The spacing of the inter-sheet distance, alternately greater and smaller than the mean, is taken to show that an arrangement Gly-Ala-Gly-Ala predominates along the polypeptide chain, with occasionally a larger unit such as serine in place of alanine. There is chemical evidence (low frequency of occurrence of glycylglycine, frequent occurrence of glycylalanine on hydrolysis) in support of this. The larger cell, which may not actually be a well-defined crystallographic cell, would accomodate large residues such as tyrosine. The authors consider that it is not necessary to relegate such residues to an amorphous phase as was done in earlier papers.

The general picture of a structure with an overall regularity in the amino-acid sequence, combined perhaps with a good deal of local variation, seems very reasonable and does not appear to the reporter to conflict with other evidence, though perhaps not all will agree with this. Although Marsh, Corey and Pauling find no need to consider an amorphous component, the evidence for such a component, based on spectroscopic observation (Ambrose and Elliott, 44; Elliott, Hanby and Malcolm, 28) is very strong. This does not, however, necessitate any hypothesis about the amino acid composition of crystalline or amorphous regions.

The interesting observation has been made that an X-ray diffraction photograph of tussah silk is almost identical with that of β-poly-L-alanine (Bamford *et al.*, 16). This must mean that the sequence gly-ala-gly-ala does not predominate in tussah; it is much more likely that a random arrangement exists. The alanine content of tussah silk is much higher than that of *Bombyx mori*.

Another silk which is of considerable interest is from the cocoon of *Anaphe moloneyi*. This silk is largely composed of alanine and glycine (58); other residues are present to the extent of 6 %. The X-ray diffraction photograph of this silk is different from either *Bombyx mori* or tussah, though all have much in common, including a very strong meridian reflection at *ca* 1.16 Å which is the 6th order of the fibre axis pseudo-repeat.

*Water-soluble silk.* — The contents of the silk-worm gland are soluble in water and the operation of spinning converts them into an insoluble form containing the proteins fibroin and seracin. The fibroin may be made water-soluble by dissolving in an aqueous solution

of various inorganic salts, followed by dialysis (von Weimarn, 59; Coleman and Howitt, 60; Ambrose *et al.*, 61). Suitable drying techniques allow a dry form of the protein to be obtained, which is soluble in water. The structure of this material is of interest, since it may resemble the structure of the fibroin component in the silk-gland.

Infra-red spectra show that water-soluble *Bombyx* silk is not in the β configuration, and it has been suggested that the polypeptide chains are in a folded (α) configuration (61). X-ray diffraction photographs suggest an amorphous structure.

It has recently been found possible to prepare the silk of *A. moloneyi* in a water-soluble form, by casting under suitable conditions from solution in trifluoracetic acid (38). The infra-red spectrum closely resembles that of α-poly-L-alanine, with some additional bands which undoubtedly are associated with glycine residues. The X-ray diffraction photograph has a strong ring corresponding to an interplane spacing of 7.0 Å, and is very probably the 1010 reflection of a hexagonally packed arrangement of α-helices (compare with α poly-L-alanine where in the powder photograph this ring (7.4 Å) is dominant). Although no oriented material has been examined, the evidence for α-helices in this water-soluble silk is strong.

If the water-soluble silk prepared as indicated above is freeze-dried from aqueous solution, a water-soluble material is obtained whose infra-red spectrum and X-ray diffraction photograph are markedly different from those obtained with silk cast from trifluoracetic acid. The X-ray photograph shows a single, diffuse ring centred at *ca.* 4.35 Å; in some samples a very faint ring at 7 Å may be seen. There is no evidence that the material contains α-helices as a major structural component, but the C = 0 stretching band at 1660 cm.$^{-1}$ is strongly suggestive of a folded structure.

Both kinds of water soluble *A. moloneyi* silk readily go over into the β form as a result of mechanical working. The aqueous solution is very unstable, much more so than aqueous solutions of *Bombyx mori* silk.

The relation between the chain configuration of native silk (in the silkworm gland) and water soluble (regenerated) silk is not known. However, Lenormant (62) has shown that dried films of these two forms of *Bombyx mori* silk have very similar infra-red spectra and they may therefore be closely related.

### *Collagen*

A very full review of what is known of the structure of collagen has been given by Bear (63) and more recent information is contained in the report of a discussion held by the Faraday Society (64).

A number of structures have recently been proposed for collagen (44, 65-69) but in no case has it been demonstrated that any of these enables an X-ray diffraction pattern to be predicted which agrees with the observed one. A considerable sharpening of the reflections in the X-ray photograph of collagen has been obtained by stretching rat tail tendon 10 % (Cowan, North and Randall, 70). This photograph resembles the diffraction pattern of a helical structure; there are no meridian reflections other than the strong reflection corresponding to 3.1 Å (2.86 Å in the unstretched fibre) which is presumably the residue repeat distance.

*Infra-red data.* — The infra-red spectrum of collagen and of gelatine has been reported in a number of papers. Although the main peptide bands occur at frequencies which show that the structure differs from that of both α and β polypeptides, they are not so different as to make it likely that the structure is based mainly on *cis* rather than *trans* amide groups (71, 72). In particular, eight-membered chelate rings formed by the hydrogen bonding of *cis* amide groups (as in diketo-piperazine) appear to be excluded as a major component. This structure is characterised by complete absence of the 1560 cm.$^{-1}$ band and by a lowered frequency of the NH stretching mode. In collagen and gelatine spectra, the 1560 cm.$^{-1}$ band is as strong as in synthetic polypeptide spectra (α and β) and the NH stretching mode is actually higher than in other polypeptides (44, 73).

If the *trans* amide group is accepted as the chief unit, some conclusions as to its orientation may be drawn from the measurements of dichroism which have been made by several authors (44, 73, 74). The relation between bond direction and transition moment for the main amide bands is now known, at least approximately (26).

The perpendicular dichroism of C = 0 and NH stretching modes, together with the fact that dichroism is greater in the former, show that the NH bond is inclined to the chain axis at an angle of more than 54° C., and that the CN bond is more nearly perpendicular to the chain axis than is the NH bond. Because of low dichroism in the 1560 cm.$^{-1}$ band (whose transition moment is approximately perpendicular to the NH bond, and in the plane of the amide group) the plane of the amide group must make a considerable angle with the chain axis.

## GLOBULAR PROTEINS

### *Introduction*

It is well known that many globular proteins may be grown as single crystals from which well-defined X-ray diffraction photographs may be obtained, and that the hundreds of crystal reflections are evidence of an ordered structure. It is perhaps not always realised that this crystallinity will be observed merely if all the molecules of the protein are identical, for they will than pack regularly; it is not at all necessary that there should be any regular periodic arrangement within the molecule. Moreover, two or more molecular species could, in some circumstances, pack regularly to form crystals. The large number of X-ray reflections observed in globular protein diffraction photographs is also, in part, a consequence of the large size of the unit cell. When considering evidence of atomic regularity, the significant thing is, not the number of reflections, but the value of the smallest interplanar distance which gives an appreciable reflection. Judged in this way, globular proteins are not markedly more regular in structure than the crystalline regions of many fibrous materials; for the purpose of structure determinations, however. they possess the immense advantage that single crystals may be obtained.

### *X-ray crystallographic studies*

*Haemoglobin.* — Lack of space makes it impossible to describe adequately the work of Bragg and Perutz on the crystal structure of haemoglobin, the recent part of which is described in ref. (75-80). Methods have now been used which allow the phases of the X-ray crystal reflections to be determined, and the projection of the electron density of the haemoglobin molecules on the *a c* plane has been calculated. No prior assumptions concerning the molecules are made, and the results are unambiguous in so far as they actually represent the projected electron density as it appears with the degree of resolution so far achieved. This, however, is too small to allow chains or haem groups to be recognised, as a single atom is smeared out to a diameter of 9 Å, and in addition the thickness of matter projected on the *a c* plane is very great. Conclusions concerning the internal structure of the haemoglobin molecule must therefore await the achievement of much higher resolution.

*Orthorhombic acid insulin sulphate.* — A good deal of interest has been aroused by the publication of Patterson sections of orthorhombic acid insulin sulphate by Low (81). These sections, it should be explained, give information on the distribution of inter-atomic vectors in the real unit cell. They do not allow an unambiguous deduction of the electron density distribution to be made. When there are regular arrangements of long molecules, however, the Patterson sections also show rod-like features (Kendrew and Perutz, 82). In the case of orthorhombic insulin, the Patterson sections suggest an arrangement of rods parallel to the *a* axis, which is 44 Å long. The β chains of Sanger's acid fraction contain 30 residues (83), which if folded in α-helices would be *ca.* 45 Å long, and this would be compatible with an arrangement of such helices parallel to the *a* axis. The stereochemistry of such an arrangement has been investigated in some detail by Robinson, and by Riley and Arndt (84, 85, 86), and the possible arrangements have been described.

Recently Sanger, Smith and Kitai (87) have established that disulphide bridges connect residues in positions $A_7$ with $B_7$, and $A_6$ with $A_{11}$. If the insulin molecule consists of two chains contained in a molecule of molecular weight 6000 (Harfenist and Craig, 88) there must be an intra-molecular cystine link, and the separation of $A_6$ and $A_{11}$ would certainly exclude the α-helix as the structure between these residues. It may be remarked, however, that the labile nature of the disulphide link makes it difficult to be certain that this link in the crystalline form is of an intra-chain type.

*Ribonuclease.* — Ribonuclease, with a molecular weight of *ca.* 13 000 and two molecules in the monoclinic unit cell has very roughly the same amount of scattering matter per unit cell as orthorhombic insulin. X-ray reflections from the wet crystal can be seen corresponding to a spacing at least as small as 1.25 Å (Carlisle, Scouloudi and Spier, 89) and the degree of order in the crystalline protein is therefore very high. If the phases of these reflections could be determined, a great deal concerning protein structure would no doubt become known. Unfortunately none of the methods used, for example, in haemoglobin, has so far been successful with ribonuclease and interpretation of the X-ray diffraction pattern has been based on Patterson diagrams (89, 90).

Carlisle and his co-workers have found an approximately hexagonal arrangement of peaks about 9.5 Å apart in the *c*-axis Patterson projection which suggests folded polypeptide chains approximately parallel to this axis. However, the detailed structure of this projection, making use of higher order reflexions than have hitherto been used in this kind of work with globular proteins, shows that the arrangement is far from possessing hexagonal symmetry. The Patterson projection does not appear to be compatible with the assumption that the rod-like structures are α-helices; they may rather be flat ribbon-like concentrations of scattering matter.

### *Discussion*

Two questions concerning the structure of globular proteins can profitably be considered at the present time.

(*a*) Are there folded polypeptide chains in globular proteins?

(*b*) If so, what is the nature of the fold?

With regard to the first question, the infra-red spectra of a number of globular proteins have C = 0 bands at *ca.* 1660 cm.$^{-1}$, *i.e.* at wave numbers which are close to those characteristic of α polypeptides (Ambrose and Elliott, 91) and quite distinct from the C = 0 bands of β polypeptides. The band characteristic of β structures (*ca.* 1630 cm.$^{-1}$) appears in the spectra of denatured proteins. X-ray investigations have revealed structures which suggest rod-like arrangements and these have been interpreted as folded polypeptide chains. Since globular proteins in the native state show no features characteristic of extended β chains, it must be concluded that some kind of folding occurs.

Concerning the nature of chain folding, little is known. We can, however, be reasonably certain that in the three proteins referred to (haemoglobin, orthorhombi acid insulin sulphate, ribonuclease) above we have not a parallel arrangement of α-helices as the main structural element. In haemoglobin, at least, the density of the rod-like features of the Patterson diagram is also much too low (Crick, 92), and in ribonuclease the details of this diagram seem to exclude the α-helix.

Infra-red dichroism has been looked for in all three crystals. In haemoglobin, Elliott and Ambrose (41) found low dichroism of the kind which would result from packing α keratin chains in the direction indicated by Perutz' rod-like structures. It is now known, however, that parallel arrangements of α-helices would give very much higher dichroic effects. In ribonuclease, somewhat higher dichroism has been found (Elliott, 93) but again much lower than would result from parallel α-helices. In this case a band was found to exhibit dichroism which is not dichroic in the spectra of α polypeptides, a result which is difficult to reconcile with the presence of α-helices in any kind of arrangement.

Orthorhombic insulin has recently been examined (Elliott, unpublished) and no dichroism has been found.

It is clear, therefore, that if the rod-like features of Patterson diagrams are polypeptide chains (which is not an inevitable interpretation in all cases) they must not have the α-helix configuration over their whole length, though portions of the chain might be in this form. Layer structures of α-helices, with different chain directions in successive layers, would give rise to low dichroism, but it is doubtful whether the absence of dichroism in insulin can be explained in this way.

It is perhaps relevant to recall that, in synthetic polypeptides with inert side chains, the α-helix is not in general the most stable form when the degree of polymerisation is small (as in insulin, with 21 and 30 residues, respectively in the A and B chains). In such cases, the β form predominates (6, 7). Although this form is not present, in the crystallographic sense, in globular proteins, the possibility of short sections of extended polypeptide chains cannot altogether be excluded by the infra-red observations. The amide groups in such portions of the chains might be hydrated, and at present the characteristic frequencies of hydrated amide groups is not known. An irregular structure (though identical in all molecules) remains a possibility. Another possibility is that there is a primary fold, different from the α-helix, possibly with local variations, and at present no grounds exist for believing that a chain configuration found in one globular protein will necessarily be found in others.

Riley and Arndt (94, 95) have compared the radial distribution function calculated from the X-ray powder diagram of bovine serum albumen with the same function calculated by Pauling and Corey for the α-helix. This function is similar to the Patterson function referred to earlier and gives information about the distribution of interatomic vectors. From the similarity of the experimentally derived and calculated functions Riley and Arndt conclude that structures closely similar to the α-helix are present in serum bovine albumen, and even that the position of the β carbon atom may be distinguished. However, the calculated function does not take into account the atoms in the side chains (except β-carbon) and refers to an isolated chain; inter-chain atomic vectors are not taken into account. Many will therefore consider that caution should be used in accepting conclusions in these circumstances.

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# Some further comments on the structure of polypeptides and proteins


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(*Received* 1 *August* 1955)

In his stimulating report on the structure of polypeptides and proteins Dr. Elliott has presented a comprehensive view of recent advances in this field. I shall restrict myself therefore to a few specific comments.

## POLYPEPTIDE CHAIN CONFIGURATIONS

The α helix structure is the most stable backbone polypeptide chain configuration with intra-chain hydrogen bonding. It has been shown to be the dominant feature of the configuration of long chain synthetic polypeptides with identical non-polar side chains. Similarly, once the coiled-coil modification is introduced the α helix structure satisfactorily describes most of the major features of the ordered regions in the α fibrous proteins. In the globular proteins, however, the α helix model needs more extensive revision and modification.

Helical configurations of peptide chains may be stable or unstable, as compared with the more randomly coiled configurations which are possible when the

$$—C = O...H—N—$$

hydrogen bonds are broken and tue regular helix structure is unfolded. The free energy of unfolding

is of course the determining factor; if this is negative the helix if it is formed at all, will be unstable. An important critical examination of the whole problem has recently been given by Schellman (1) in terms of the α helix configuration. Heat is absorbed when a —CO...NH-bond is broken. In a medium of low dielectric constant, incapable of hydrogen bond formation with the amide CO.NH groups in the peptide chain, $\Delta H$ the heat absorbed, is of the order of 7 or 8 kcal./mole of such bonds broken. In water, on the other hand, the net value of $\Delta H$ for the reaction, which may be considered as hydrogen bond interchange rather than hydrogen bond breakage, is naturally much less than for the other. Schellman estimates a value of 1.5 kcal./mole per bond, from data on the association of urea molecules in aqueous solution and the temperature coefficient of the association constant.

A regular hydrogen-bonded chain configuration has low configurational entropy. The gain in entropy on unfolding will contribute a negative term to the free energy of unfolding. Schellman has calculated that in general the free energy of unfolding per residue $\Delta F_{res}$ of a hydrogen bonded peptide structure at room temperature, neglecting side chain interactions, is of the order of $RT$ or less, that is about 500 ± 100 cal.

Donohue (2) has studied the relative stabilities of a group of several helices which includes the first four members of the 'α' series :

$$\begin{array}{l} \mathrm{H}\ldots\ldots\ldots\ldots\ldots\ldots\ldots\mathrm{O} \\ \;| \qquad\qquad\qquad\quad\;\; | \\ \text{—N(CH.CO.NH.)}_n\text{C—} \end{array}$$

where n = 1, 2, 3 and 4. The α helix structure (n = 3) shows excellent van der Waals packing across the interior of the coil; none of the other three helices pack so well. Donohue has not included the contributions of van der Waals forces in his estimates of the order of relative stability within the group. Even without this he finds that the two most reasonable alternative structures, the π (3) helix (n = 4) and a ribbon structure (4) 2.27 (n = 1), are less stable than the α helix by an amount which is approximately equal to 500 cal./residue/mole. It is perhaps significant that the free energy of unfolding per residue calculated by Schellman is of the same order of magnitude as the differential relative stability of the α helix compared to the other most reasonable alternative structures. From this study he has shown that there will be a critical chain length below which the helical configuration is unstable. This critical size has been estimated as probably between 8 and 15 peptide residues for a polypeptide chain in aqueous solution, but this calculation neglects side chain interactions.

### *Globular proteins*

In globular proteins the stable discrete unit is a single molecule. Ordered and disordered regions of chain configurations are confined within a single molecule and establish the unique three-dimensional molecular structure. Globular proteins are almost always shorter than the model structure which would be derived if peptide chains of average length (calculated from end group determinations) were each coiled into a single cylindrical α helix structure and then stacked together in close packed array. The peptide chains must therefore turn corners and reverse direction. For hemoglobin, as was reported at the last International Congress, Bragg, Howells and Perutz (5) and Crick (6) have both shown that this model is oversimplified. The regions of α helix configuration must be shorter than the distance between two turns and the parallel packing of the rods less perfect than in the most simple picture. The lengths of coiled chain may zigzag about the overall axial direction. Thus in globular proteins the regions in which the specific molecular configuration is not associated with any regular peptide chain configurations are probably more numerous and extensive than the number of complete 180° turns. In some parts of the molecule, therefore, other types of intra-molecular interactions must predominate over those which lead to an α helix. These will determine the local configuration. Apart from the intra-chain amide group interactions (hydrogen bonds) of a coiled chain, three other types of interactions are possible :

(*a*) Inter-chain amide hydrogen bonds. Ordered regions with regular inter-chain hydrogen bonds are regions of β-configuration. There is no evidence for this type of bonding in globular proteins.

(*b*) Side-chain side-chain interactions. Both inter and intra-chain covalent (*e.g.*, cystine -S-S-) linkages are known to exist. Electrostatic interactions and interactions between adjacent non-polar side chains will depend upon the distribution of polar and non-polar groups.

(*c*) Side-chain main-chain interactions are possible, for example hydrogen bonds may be formed between polar and hydroxyl containing side chains and peptide chain amide -CO.NH-groups.

In brief, the specific molecular configuration will be determined in part by the specific amino acid sequences and consequent intra-molecular interactions. Since major discontinuities do certainly exist in globular proteins we must conclude that some amino acid sequences inhibit the formation of regular backbone chain configurations.

### *Side-chain interactions*

Proline residues introduce a stereochemical discontinuity in a regular helical chain configuration. In left-handed α helices a proline residue markedly alters the chain direction. It has been suggested that proline residues may provide a corner turning mechanism which causes regions of coiled polypeptide chain to fold back on each other.

In both hemoglobin and ribonuclease the proline residues are numerically almost adequate for this task. The proposal demands a specific sequential distribution of proline residues along the polypeptide chains of these proteins. However, Hirs, Stein and Moore (7) have shown recently that in the ribonuclease molecule, 3 of the 5 proline residues are in the short terminal segment of 22 residues. There are 126 residues in the chain and the turns must occur at more nearly regular intervals to give a relatively compact molecular shape.

Lindley (8) has made model studies of the side-chain side-chain and side-chain main-chain interactions which

could take place if certain specific hypothetical amino acid sequences were to occur along an $\alpha$ helix. These would involve a region of disturbance in the $\alpha$ helical configurations. He has suggested mechanisms for 180° turns and a rather different role for proline. When an L-proline residue is incorporated into a left-handed $\alpha$ helix it can initiate a change of hand LH $\rightarrow$ RH in the helix. The postulated structure is stabilized by using an arginine side-chain residue to bond the main chain carboxyl groups which cannot form intra-chain hydrogen bonds. There is no change in direction of the helical axis.

Lindley has also considered the role of intra-chain cystine disulphide linkages in promoting a LH $\rightarrow$ RH changeover. A detailed model for this sense change has been devised by Lindley and Rollet (9) for insulin.

All the mechanisms referred to so far have involved a LH $\rightarrow$ RH sense change, the RH $\rightarrow$ LH changes must also take place, and Lindley has suggested an appropriate amino acid sequence. The broad implications of this study, the possible side-chain participation in the stabilization of local regions of chain, are of greater significance than the details of the particular amino acid sequences proposed.

## GLOBULAR PROTEINS

The X-ray crystallographic studies of hemoglobin by Perutz and his co-workers have made rapid and stimulating progress. One finding of Bragg and Perutz (10) should be of especial interest here. Work on mercurial derivatives, which give crystals isomorphous with those of the original hemoglobin, indicates that the two pairs of sulfydryl groups are arranged symmetrically around the two-fold axis of the hemoglobin molecule. Harker and his co-workers (11) have prepared a large number of new crystalline modifications of ribonuclease. Outstanding advances in technique have been made by this group. Fourier analyses of heavy atom derivatives are in progress and should soon lead to significant structural information for ribonuclease.

### *Insulin*

The molecular size of insulin was established by Harfenist and Craig (12). The unit of packing in the orthorhombic crystals of acid insulin sulfate is the solution monomer containing 2 (A and B) molecules (13). The peak distribution in the vector structure (Patterson series) suggests that each 12 000 unit may be made up of four cylindrical rods of coiled polypeptide chain stacked in close-packed array parallel to the *a* axis. From calculations based on the length and cross-packing area of each rod, the density, and the number of residues in the B chain, it appears that an $\alpha$ helix might be the most plausible model for the chain configuration (14, 15).

The $\alpha$ helix structure satisfies the simple geometrical packing requirements of the unit cell. An early model study based on the Sanger amino-acid-sequences established that in the two chain (A + B) unit a simple model of parallel $\alpha$ helices for both A and B chain required modification. The -C($\beta$)-S-(half cystine) residues on the $A_6$ and $A_{11}$ $\alpha$ carbon positions are too far removed in space on a simple $\alpha$ helix structure to permit the formation of the $A_6^{11}$ intra-chain disulphide bond established by Sanger, Smith and Kitai (16).

Both the two terminal parts of the A chain and the whole of the B chain may be in the $\alpha$-helix configuration without affecting the inter-chain cystine linkages. If the specific configuration of the pentapeptide loop is irregular, then intra-chain hydrogen bonds between peptide CO.NH residues cannot be formed. The problem of whether there is any configuration for this region of the chain which would permit some intra-chain hydrogen bonding, or whether it is possible to maintain the $\alpha$ helix configuration in at least some part of the $A_6^{11}$ loop, can be studied using scale atomic models.

Lindley and Rollett (9) have shown that the chain in this region may be twisted into a more regular configuration if it joins two segments of a chain which have different screw directions. If the $A_{1-9}$ part of the chain is a left-handed coil and the $A_{9-21}$ part is a right-handed coil, then the $A_6$–$A_{11}$ region forms the figure of eight required by this change of hand in the coil. In the detailed model, which they have developed, only two of the main chain NH...OC hydrogen bonds are broken. The CO group of residue 8 could, in beef insulin, form an OH...O bond with the hydroxyl group of the seryl residue 9. In this model the helical axis of the right-handed $A_{9-21}$ sequence is parallel to a right-handed B chain $\alpha$ helix. The helical axis of the $A_{1-7}$ segment is in the same plane as the B and $A_{9-21}$ axes; it is tilted away from B at an angle of 30°.

Low (17) has constructed a predominantly left-handed model using a similar change of hand from a left-handed $A_{1-8}$ segment to a right-handed $A_{11-21}$ segment. The B chain in her model is a left-handed $\alpha$ helix. In this structure the two parts of the A chain do not lie in the same plane. Three inter chain hydrogen bonds are broken in this model. Both structures are plausible. The Lindley and Rollett model, which is predominantly right-handed, is the more satisfying solution of the simple stereochemical problem: to find a model structure for the insulin molecule in which all of the B chain and as much as possible of the A chain are in the $\alpha$ helix configuration. Arndt and Riley (18) however regard the $\alpha_1$ left-handed helix as the most plausible predominant chain configuration in insulin.

Both the Low and the Lindley and Rollett models can be stacked in close packed array. Lindley and Rollett have given a detailed discussion of the possible modes of inter-molecular packing in the rhombohedral crystals of zinc insulin. They suggest that the 12 000 unit which is the stable monomer in solution may be made up of 2 (A + B) units packed in rectangular array and held together by specific interaction between zinc ions and histidine and glutamic acid residues. Zinc is not, however, necessary to hold the 12 000 unit intact and other packing models can be devised.

The important question is the revelance of these stereochemical models to the actual insulin structure. The X-ray study of acid insulin sulfate crystals offers rather persuasive geometrical evidence that some part at least of the insulin molecule is in the $\alpha$ helix configuration. Unfortunately, despite intensive search in X-ray photographs of wet crystals of acid insulin sulfate, the 1.5 A spacing characteristic of the $\alpha$-helix structure has not

been found. It is a little difficult to reconcile the studies of Elliott (19), using polarized infrared radiation, on air-dried crystals of acid insulin sulfate with an extensive α helix structure in insulin.

When the cystine linkages in insulin are oxidized the A and B chains both appear to be in the β configuration (18). Hvidt and Linderstrom-Lang (20) have studied the kinetics of the deuterium exchange of insulin with $D_2O$. Linderstrom-Lang (21) reports that a certain number of the hydrogen atoms exchange very slowly. The actual number of slowly exchanging hydrogen atoms would suggest that only those regions of the A and B chains between the two inter-chain cystine linkages are maintained in a coiled configuration with intra-chain hydrogen bonds.

Even for insulin, therefore, in which the chain sequences and cross links are completely known, the exact spatial configuration of the molecule still remains in doubt. Further crystal structure studies of insulin are in progress in this laboratory.

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# The connection between the chemical structure and the contraction-relaxation process of collagen fibre


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(*Received* 24 *March* 1955)

Recently we gave a report (1) on the chemical contraction-relaxation process of collagen fibres which takes place in 40 % potassium iodide at 20° C. One of us (2) has shown that the collagen fibres consist of two separate protein components which differ from each other in physicochemical, polarization-optical and enzymological properties. One of these is the procollagen (3) and the other is the insoluble component of collagen which we call metacollagen. Similarly to actomyosin collagen is made up of procollagen and metacollagen and the phenomenon of contraction-relaxation can only be noticed if the connection between the two components exists.

In the process of contraction-relaxation changes occur which depend on the alteration of the chemical composition of collagen fibres. The contraction-relaxation process is 'normal' if the rapid contraction of the fibres is followed by quick relaxation. Collagen fibres of young animals show normal contraction-relaxation while the 'old' ones only contract but do not relax or only to a small degree. The contraction process of collagen was successfully affected by procedures which cause already known alteration in the chemical structure of collagen. The following findings were established :

(*a*) After the extraction of procollagen from collagen fibres with citrate buffer at pH 4 there is no more contraction. It is concluded from this fact that procollagen plays an important role in the stabilisation of collagen fibres.

(*b*) The extraction of mucoproteids with $CaCl_2$ results in a rapid contraction of a 'young' fibre but without following relaxation. The 'young' fibres can thus be transformed by $CaCl_2$ to 'old' fibres.

(*c*) The treatment of collagen fibres with hyaluronidase in water solution instead of acid solution at pH 5.2 (to free the fibres of chondroitin-sulphate and hyaluronic acid) thus avoiding dissolution of procollagen, causes the process of contraction to take place in a small degree and the fibres to break during relaxation. But the whole contraction-relaxation process can be completed

contrary to the procollagen-free fibres in which the contraction-relaxation process can not be carried out.

(*d*) The contraction of the fibres will be stronger and quicker under the influence of elastase but during relaxation they get torn. According to the experiments of Hall (4) and of Banga (5) elastase from a complex enzyme system. One components of this system hydrolyses one of the mucoids of collagen. It is possible that this mucoid also plays a part in the stabilization of collagen fibres.

It has been proved by these experiments that, as Jackson (6) also suggested, several factors play a part in the stabilization of collagen and besides the chondroitin-sulphate some other mucopolysaccharides or mucoproteins are concerned.

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# Investigations of dissolved proteins by means of the X-rays small-angle method

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(*Received* 9 *September* 1955)

## INTRODUCTION

The theory yields the following, shortly summarized results on the small-angle scattering of X-rays especially of a homodisperse diluted solution :

(*i*) The intensity of diffraction at zero-angle is a direct measure for the volume of the particles. Measurements of absolute intensities in the X-ray field, however, are frequently exposed to relatively large errors. This shortcoming can be overcome by making use of the invariant $Q$ as developed in this laboratory by G. Porod (1). It is defined as :

$$\bar{Q} = \int_0^{\infty} \bar{I}.x.dx \qquad (1)$$

$\bar{I}$ is the intensity as measured in a set up with slit-collimation. The collimation error however can be eliminated (2) and so we get $I$, the slit-corrected intensity. $x$ is the distance from the center of the densitometer curve. It is related with half the scattering-angle $\delta$ by :

$$\delta = \frac{x}{2\,a.p} \qquad (2)$$

$a$ = distance specimen-film ; $p$ = ratio of enlargement.

It can be shown, that the invariant $\bar{Q}$ depends merely on concentration and electron density difference, not however on the degree of dispersion. Since, on the other hand for a given concentration the collimation corrected zero-angle intensity $I_0$ increases with particle weight (molecular weight) the quotient $I_0/\bar{Q}$ is a measure of the molecular weight, resp. the volume $V$. $I_0$, thereby, need not be measured absolutely, but is to be plotted in the same scale as the intensity used for calculating $\bar{Q}$. We can write (3) :

$$V = \frac{I_0}{\bar{Q}} \frac{(\lambda.a.p)^3}{2\,\pi^2} \qquad (3)$$

where $\lambda$ = wave-length = 1.54 Å.

(*ii*) The shape of the scattering curve approximates according Guinier (4) a Gaussian curve at small angles :

$$I = I_0.e^{-KR^2\delta^2} \; ; \; K = \frac{16\,\pi^2}{3\,\lambda^2} \qquad (4a)$$

$R$ represents the so-called radius of inertia, that is the square root of the average square distance of all electrons of the particle from its centre of gravity.

From (*4a*) follows :

$$\ln I = \ln I_0 - K\,R^2\,\delta^2 \qquad (4b)$$

If we plot $\ln I$ *vs.* $\delta^2$ we obtain accordingly for the end tangent :

$$\mathrm{tg}\,\alpha = -K\,R^2 \qquad (4c)$$

so that we can take off it directly the radius of inertia. Introducing (*2*) in (*4b*) we find :

$$R = 0.64\,\sqrt{\mathrm{tg}\,\alpha}\,(a.p)\,\text{Å} \qquad (4d)$$

(*iii*) For a given molecular weight (volume) $R$ is the bigger, the more the shape deviates from a sphere. By comparing the radius of inertia $R$ as determined experimentally with that calculated from the known molecular weight by assuming the shape of a sphere, $R'$, we obtain a shape factor.

$$f = \frac{R}{R'} \qquad (5)$$

This, however, does not yet allow to fix unambiguously a shape type, since a certain shape factor may be realized for example likewise with an elongated or a prolated ellipsoid.

(*iv*) The further development of the theory has shown, that the scattering curve exactly corresponds to a Gaussian curve only in its innermost part. In the outer part typical deviations occur, which are varying from shape to shape (5-7). By measuring those deviations

experimentally one can make decisions within the different shapes compatible with the shape factor $f$.

($v$) Great practical importance is connected with the case of particles which are extremely elongated in one direction. We could show (5, 7), that the scattering curve then can be split into a length-factor and a cross-section-factor.

$$I = I(l).I(q) \qquad (6)$$

The length-factor $I(l)$ is simply given by $1/x$. Therefore, by multiplying the scattering curve by $x$ the length-factor is eliminated and the cross-section-factor is obtained. If the latter is plotted according to Guinier, the slope of the end tangent yields a radius of inertia $R_q$ which however now belongs to the cross section. By means of zero intensity $(I.x)_0$ of the cross-section factor and the invariant $\overline{Q}$ a determination of the area of the cross section is possible in a way analogous to that, which yielded according (3) to the volume of the corpuscular particles. The relation holds (1,3) :

$$q = \frac{(I.x)_0}{\overline{Q}} \cdot \frac{(\lambda.a.p)^2}{\pi^2} \qquad (7)$$

To a certain area now corresponds a smallest possible radius of inertia, namely that one under assumption of circular shape, $R_q'$. The quotient $R_q/R_q'$ again yields a shape factor of the cross section. The deviation of circular shape turns out in the outer part of the curve, which provides an independent means for determination of the shape of the cross-section.

($vi$) If the particles are not infinitely long, but one dimension is considerably longer than the two other ones, all that has been said for the infinitely long particles remains still valid, if only the scattering at the very smallest angles is excluded from the discussion. If additional data at sufficient small angles are available, the radius of inertia of the whole particle is determinable and therewith its length.

## RESULTS

Some characteristic results shall be reported, which have been obtained in the way outlined above in our labs in the last time.

### *Gamma-globuline* (3)

The curve, rising with about $1/x$, suggests the assumption of an elongated shape. The invariant $\overline{Q} = 3.80$ cm² and the zero intensity of the cross-section factor $(I.x)_0 = 0.256$ (figure 1) yield according (7) a cross section area of $q = 866$ Å². On the other hand, the radius of inertia of the cross section $R_q$ from figure 1 is 15 Å. If assuming an elliptical shape of the cross section, we have :

$$q = 866 \text{ Å}^2 = a.b.\pi \text{ and } R_q^2 = 15^2 = (a^2 + b^2)/4 \qquad (8)$$

From these two equations we readily read off :

$$a = 9.7 \text{ Å and } b = 28.4 \text{ Å}$$

This axial ratio could be veryfied also by the shape of the scattering curve at higher angles. Thus we have

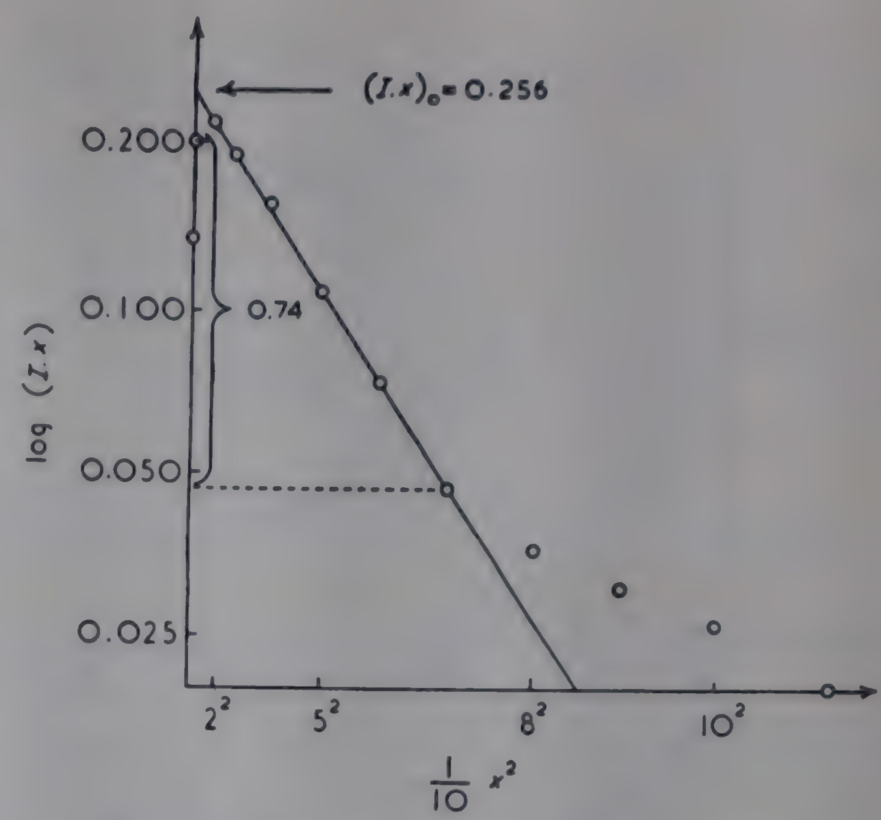


FIG. 1. — Cross-section-factor $I.x$ of γ-globulin.

been led to the assumption of the shape of an elliptical cylinder.

The course of the whole scattering curve at smallest angles yields a radius of inertia of $R = 70$ Å. For an elliptical cylinder we can readily calculate the length $l$ by means of $R$ and $R_2$ :

$$l = \sqrt{12\,(R^2 - R_q^2)} = 236 \text{ Å} \qquad (9)$$

This consideration, based on the splitting into length- and cross-section-factor, can be controlled and improved by means of the whole scattering curve for elliptical cylinders, if the scattering at smallest angles is included. We shall find out about the shape by means of two series of comparisons (8) : ($a$) variation of length with fixed cross-section, and ($b$) variation of cross-section with fixed length.

With elongated particles variation of the length at fixed cross-section is born out, above all, at small scattering angles, while the behaviour at large angles remains practically unaltered. With a fixed ratio of the cross-section axes of 1:3, the ratio of the length $l$ to the 'equivalent radius' $\bar{r}$ of the cross-section (*) has been varied in the neighbourhood of the suspected value, which has been doubtlessly obtained approximately correct in the above consideration. Figure 2 shows, that for $h : 2\bar{r} = 5.5$ obviously a very exact correspondance exists between experiment and theory, so that this value can be regarded as reliable.

(*) We understand thereby the radius of that circle, which has the same radius of inertia as the cross-section. For an ellipse with axes $a$ and $b$ it is given by the relation :

$$r = \sqrt{\frac{a^2 + b^2}{2}}$$

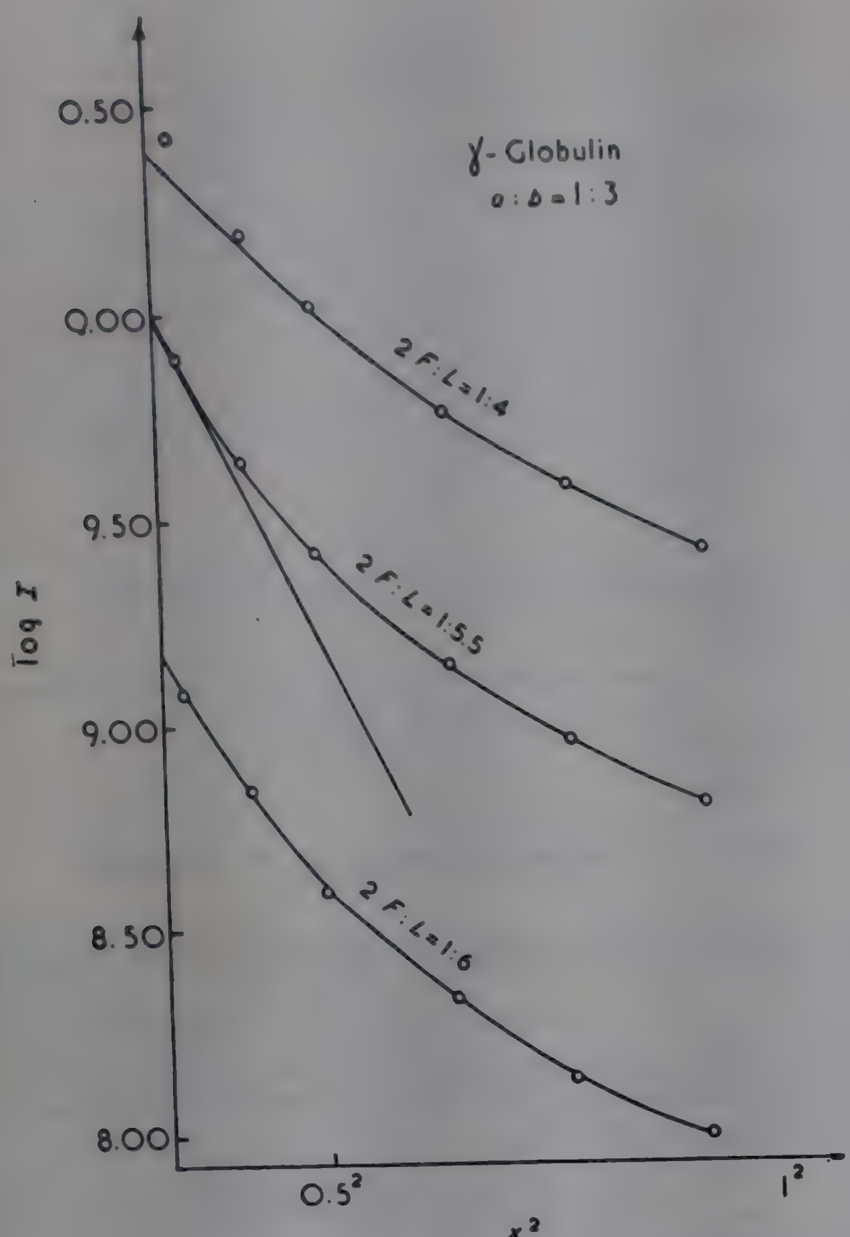


FIG. 2. — Scattering-curve of γ-globulin (points), compared with theoretical curves for elliptical cylinders of varied length.

The theoretical scattering curves (*) are normalized in such a way, that corresponding values of $x$ of both the theoretical curve and the experimental curve are connected by the relation :

$$\frac{x_{\text{exp.}}}{a \cdot p} \cdot \bar{r} = x_{\text{theor.}} \cdot \frac{\lambda}{2\pi} \qquad (10)$$

In order to adapt the experimental points to the theoretical curves the $x_{\text{exp.}}$-values had to be multiplied by a factor of 0.37, that is :

$$x_{\text{theor.}} = x_{\text{exp.}} \times 0.37$$

From this we obtain by means of (*10*) :

$$\bar{r} = 21 \text{ Å}$$

and :

$$l = 5.5 \times 2\bar{r} = 231 \text{ Å}$$

With fixed length and varied cross section, differences are to be expected only at larger scattering angles.

(*) They are calculated according Porod (3), who was supported by Sacken.

Figure 3 shows, that only with a ratio of the cross-section axes of 1:3 agreement can be attained at. From :

$$\bar{r} = 21 \text{ Å} = \sqrt{\frac{a^2 + b^2}{2}}$$

we presently find for $a = 3\,b$ :

$$b = 9.4 \text{ Å} \qquad a = 28.2 \text{ Å}$$

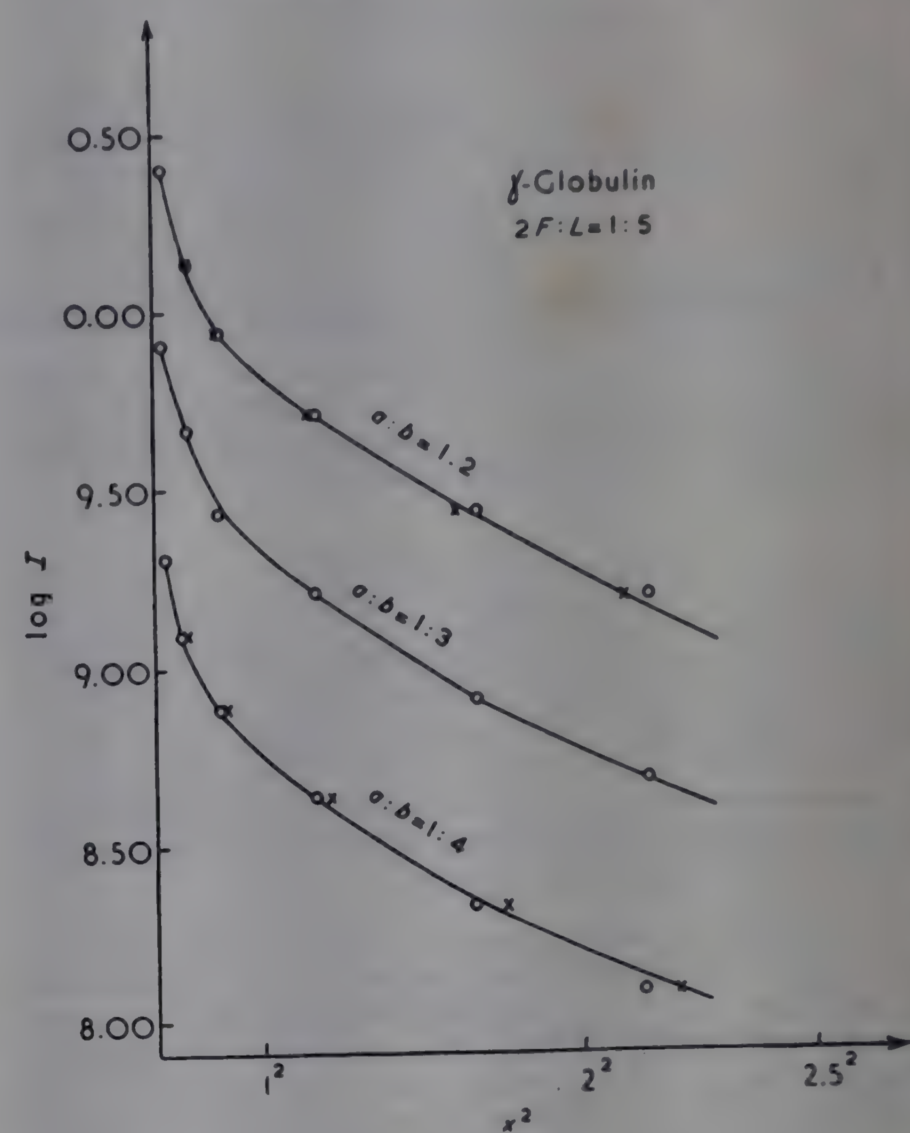


FIG. 3. — Scattering-curve γ-globulin (points), compared with theoretical curves for elliptical cylinders of varied cross-section-shape.

$l$, $a$ and $b$ agree satisfactorily with the values obtained by the splitting up into length- and cross-section factor. We then find :

$$V = a \cdot b \cdot \pi \cdot l = 1.93 \cdot 10^5 \text{ Å}^3$$

and with :

$$\sigma = 1.354 \qquad (9)$$

$$M = 1.57 \times 10^5$$

This X-ray molecular weight agrees very exactly with those obtained from newer ultracentrifuge-measurements (10), $1.56 \times 10^{-5}$. The length of 231 Å as calculated by us is practically identical with the value from viscosity (11) and streaming-birefringence (12) of 230 Å. With the measurements other than small angle scattering, however, no possibility did exist to arrive at somehow closer results on the shape of the cross section. The X-ray small angle analysis, therefore, turns out to be a means to determine the shape of dissolved particles more precise and detailed than any other method.

TABLE I

*Particle sizes and forms of the renatured silk* (20)

| | Sample | Form type | $R_q$ | Thickness | $q$ from $(I\,x)_0$ and $Q$ | Axes $a$ and $b$ from $q$ and $R_q$ | $a : b$ | $Q$exp./$Q$theor. |
|---|---|---|---|---|---|---|---|---|
| 1 | 22 % gel in water . . . . . . . | rodlet | 31.2 | | $5.3 \times 10^3$ | $58 \times 92$ | 1 : 1.58 | 0.60 |
| 2 | 10.34 % gel in water . . . . . . | » | 31.8 | | $5.48 \times 10^3$ | $59 \times 93$ | 1 : 1.59 | |
| 3 | 13.8 % solution in water . . . . | » | 33 | | $6.23 \times 10^3$ | $67 \times 93$ | 1 : 1.37 | 0.10 |
| 4 | 15.8 % solution in 0.5 N lye . . . | » | 32.5 | | $6.14 \times 10^3$ | $68 \times 90$ | 1 : 1.31 | 0.03 |
| 5 | 20 % gel → alcohol → dried . . . | » | 32 | | | | | |
| 6 | 20 % gel → benzole → dried . . . | » | 55 | | $10.9 \times 10^3$ | $61 \times 181$ | 1 : 2.99 | 0.58 |
| 7 | 8.8 % solution in water + 3.27 % KCl | » | 19.6 | | $1.23 \times 10^3$ | $65 \times 19$ | 1 : 3.42 | |
| 8 | 4.1 % gel in water . . . . . . . | lamella | | 25 — 50 | | | | 0.62 |

### *Silk fibroin*

*Fresh silk-gel.* — From the fresh gel contained in the silk-gland of mature silk worms a small angle diagram could be obtained (13). Multiplication with $x$ yielded a horizontal line, that is the scattering curve response followed $1/x$. From this we concluded elongated, thin particles. Measurements of the absolute intensity allows the calculation of scattering mass per unit length of these particles. From this we found, that one amino-acid residue occupates a region of 1.4 Å. This value agrees with that to be expected for a α-helix according to Pauling-Corey (14). We therefrom conclude, that in the solution probably single silk-molecules prevail in the shape of a α-helix. This result is surprising, since the silk-thread represents a β-protein. But Bamford, Elliot, Ambrose, Toms, and Hanby (15) too have suspected the occurrence of the α-form from their IR-measurements on fresh silk films. Obviously the transformation into the β-form takes place only in a later stage of the thread formation.

*Aged silk-gel.* — Two types of particles exist together : a rather spherical one with a radius of inertia $R > 32.5$ Å and a molecular weight higher than 280 000 and another one of the shape of a short cylinder with a molecular weight of about $21 \times 10^6$.

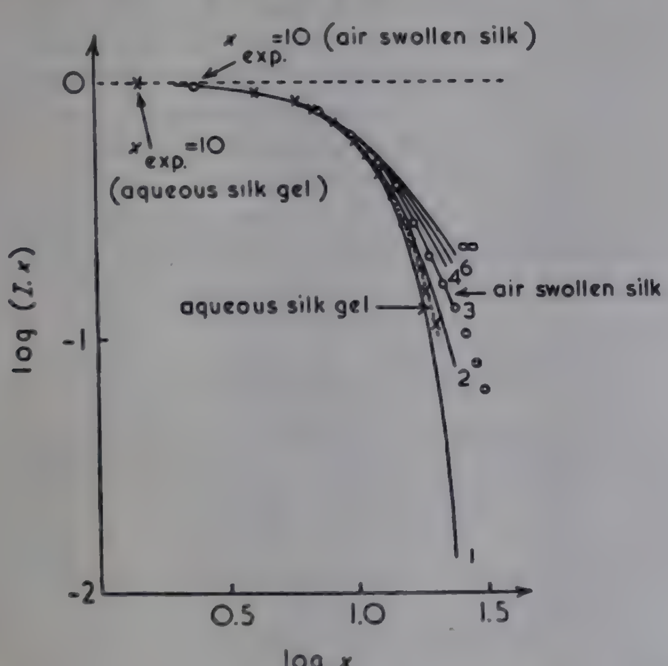


FIG. 4. — Cross-section-factors $(I.x)$ of aqueous 22 % silk gel and air swollen silk gel in a double logarithmic plot (points), compared with theoretical curves for varied axes ratios.

*Renaturated silk-gel.* — If fibrous silk is dissolved according to the procedure of Coleman and Howitt (17), the solution obtained gels after some time. The small angle analysis shows, that very alongated particles have formed (18, 19). Depending on sample history these particles may possess different cross sections (figure 4 and table I). The accuracy of these values, for reasons not being discussed here, is not very high. However, a striking regularity is observed : one dimension of the cross section is always given by about 60 Å, while the other dimension probably represents multiples of about 18 Å (namely 18.90 and 180 Å).

Much work still to come will be required in order to clear up the inner connections of this multitude of particle-shapes for silk. But here too, the small angle method proves to be a superior means, to which we do owe the knowledge of the existence of these shapes at all.

The investigations reported here, have been carried out with a new technique, developed two years ago in our laboratory (20).

I am very much indebted to the Rockefeller-Foundation for providing financial support.

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# The structure of collagen : recent observations and new models

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(*Received* 1 *August* 1955)

This report on the structure of collagen is by no means intended as a complete review, and cannot be so within the limited time which is available. Rather is it intended to select certain features for discussion, relating, where possible, the contributions from different fields. The terminology of Schmitt *et al.* (1) and Bear (2) will be adopted in describing the different structural levels : in decreasing order of magnitude 'fibre', 'fibril', 'filament', 'protofibril', the latter being the unit chain of diameter 12 Å based on X-ray measurements of dry fibres.

*Periodic structure along the fibril, the fundamental unit*

Collagen fibrils from tendon or skin show, in the electron microscope, a characteristic banded structure with average period 640 Å (1). Along the fibril denser (bands) and less dense (interbands) regions alternate, and although, within limits, the overall period is constant, a wide variation in the size and number of bands and interbands is found in different fibrils, even when these are from the same source (3). The 640 Å axial periodicity is also exhibited by X-ray diffraction of collagen fibres at small angles (4). Although differences are found in the low angle patterns of material from different sources, the intensity distribution among successive orders of the main period appears to be relatively constant for specimens from the same source (*e.g.* rat tail tendon). This suggests a constant distribution of fibrils with different band-interband arrangements.

A study of line shape for the various orders of diffraction, $k$, has led Bear and Bolduan (5) to the conclusion that regions of structural perfection and imperfection alternate along the axis. They suggest that these correspond respectively to the interbands and bands, and that the denser bands contain a preponderance of large side chains. Some support for this theory has come from the effects of P.T.A. (6), water (7), and tension (8) on the low angle pattern.

A more direct attempt to relate the results of X-ray diffraction and electron microscopy has been made recently by Burge and Randall (9). These authors have measured the density fluctuations along the fibril axis in several hundred electron micrographs of unstained, unshadowed specimens from both fowl neck and rat tail tendon. The observed electron density distribution, $N(v)$, for the most detailed fibril (largest number of bands and interbands) was used to calculate the structure factor, $F_k$, for the first ten orders, $k$, by numerical integration of the expression (10) :

$$F_k = \int_0^1 N(v) . \exp (2 \pi i k v) \; dv$$

A comparison of the calculated $|F_k|$ with the square roots of the observed intensities showed $|F_k|$ too high at large values of $k$. If, however, the independent contributions from all structural types from the most detailed to the least detailed were included, remarkably good agreement between observed and calculated values was obtained. Mathematically this process is equivalent to modulating $|F_k|$ for the most detailed by an 'artificial temperature factor'. Thus it appears the most detailed fibril represents the fundamental electron density distribution (possibly that of the protofibril), less detailed fibrils having the same elements, but with their corresponding density regions in less exact register.

The length of the structural unit repeating along the fibril axis is still uncertain. On the basis of observations of long spacing (2000 Å) fibrils and segments, Gross *et al.* (11) have proposed a fundamental particle, 'tropocollagen', about 2000 Å in length. Recently Boedtker and Doty (12) have obtained monodisperse solutions of citrate extracts of ichthyocol containing a particle of molecular weight 300 000 and dimensions 2900 Å by 14 Å (measured by a number of different physical methods). They suggest that this may be the 'tropocollagen' molecule. On the other hand the 'sub-unit' of length 210 Å of North, Cowan and Randall (13) appears from the calculations of Burge and Randall (9) to be more apparent than real.

### *Structure transverse to the fibril axis*

Physical measurements on collagen solutions have shown that although the particle length may vary widely with conditions, the width is normally that of a single protofibril. Under certain conditions, however, evidence has recently been obtained for the presence of lateral dimers (12) and trimers (14).

More definite evidence of lateral aggregation has been obtained by X-ray diffraction of adult rat tail tendon kept in its native moist condition (13). Reflections are observed on and near the equator with spacings greater than those expected for a simple hexagonal system of protofibrils. The actual spacings observed vary to some extent with different specimens. This is probably the result of slight differences in packing of the large units. At present the exact interpretation of the large spacings is not clear, but there is some indication of a unit of about 150-200 Å in diameter. Possibly a range of sizes of protofibrillar bundles occurs either at a single level or at different levels along the fibril length. The intensity distribution along the equator resembles that of a limited number of cylinders on a hexagonal lattice. If the fibres are dried and rewet, however, the maximum shifts to larger spacing, consistent either with a breakdown in structure or with greater swelling, but discrete reflections are not obtained. The latter are also destroyed by tension and by acid swelling. The occurrence of off-equatorial maxima may be due to longitudinal displacements in either a regular or irregular manner.

Direct evidence of a structural level intermediate between that of the fibril and the protofibril has come from electron micrographs of thin cross-sections of fowl metatarsal tendon stained with osmium (15). Adult tendon has an average fibril diameter of 750 Å, but within the fibril cross-section a number of smaller units are visible. The exact size, number and mode of packing of these units is not as yet clearly defined, but there appear to be four or five of them across the diameter. The filament, or 'sub-fibril' diameters are therefore 150-200 Å across if close-packed. The fibrils themselves are on a roughly hexagonal array, but are not close-packed. Both the fibril diameter (80 Å in early embryonic tendon) and the packing fraction of the fibrils within the bundle (fibre) increase continuously with age. The former observation may be taken as evidence against a tubullar fibril structure, as may the comparatively uniform density of the fibril cross-section. Whether or not any changes in cross-sectional structure occur between bands and inter-bands is an interesting point yet to be determined.

### *The structure of the protofibril*

That the high angle X-ray diffraction pattern of collagen fibres supports a helical configuration for the polypeptide chains has been pointed out by a number of independent workers (16, 17, 18). In the present discussion this will be taken for granted. Although it is possible theoretically to fit an infinite number of helical chain systems (single and multiple) to the helical net pattern (19), it seems more likely that the structure will correspond to a small number of simple alternatives (8). A study of the intensity distribution in prominent layer lines (20) has led to the conclusion that the most probable system is either a single chain with pitch $\sim$10 Å, unit repeat $\sim$3 Å and three (or four) non-equivalent amino acid residues in the repeating unit, or an arrangement of three (or four) non-coaxial helices each with pitch $\sim$10 Å and residue repeat $\sim$3 Å. The difficulties of systematic model building in the former system are illustrated by the fact that even if only most favoured configurations of bonds at the $\alpha$-carbon atom are adopted (21), there are over 45 000 possible arrangements of three non-equivalent residues. In the second arrangement the main difficulty is in forming an adequate sytem of hydrogen bonds (17).

Little is yet known of the sequence of amino acid residues along the polypeptide chain. It appears from recent analyses, however, that the juxtaposition of L-proline and hydroxy-L-proline may be of not infrequent occurrence in the sequence (22, 23). Such results should be taken into account in model building. The analysis do not favour a simple sequence pro-gly-R throughout.

A structure has recently been proposed for poly-L-proline (24). This is a three-fold spiral of planar trans residues, with pitch 9.36 Å and residue repeat 3.12 Å. When unwound slightly this chain can take up a configuration fitting that of the single non-coaxial helices mentioned above. A model composed of three such chains joined by lateral hydrogen bonds in a complex system of three coiled coils has recently been described by Ramachandran and Kartha (25). There is reason to hope that this may represent a close approach to the structure of the collagen protofibrils.

The word 'sub-fibril' is suggested rather than 'filament' to denote what appears to be a definite sub-unit. Filaments observed at the frayed ends of collagen fibrils are of variable size.

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# Essai d'interprétation de quelques propriétés physico-chimiques des solutions étendues d'acide désoxyribonucléique (*)

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(*Reçu le 2 mai* 1955)

## INTRODUCTION

Au cours de ces dernières années, un travail considérable a été accompli pour séparer et identifier les constituants du noyau cellulaire.

Tout particulièrement ont été proposées un certain nombre de méthodes d'extraction de l'acide désoxyribonucléique dont les chimistes nous ont appris que la molécule de masse moléculaire élevée contenait des séquences du type :

$$\begin{array}{ccccccccccccccc} & \mathrm{O} & & & & & \mathrm{O} & & & & & \mathrm{O} & & \\ & \| & & & & & \| & & & & & \| & & \\ - & \mathrm{P} & - \mathrm{O} - & \mathrm{S} & - \mathrm{O} - & & \mathrm{P} & - \mathrm{O} - & \mathrm{S} & - \mathrm{O} - & & \mathrm{P} & - \mathrm{O} - \\ & | & & | & & & | & & | & & & | & \\ & \mathrm{OH} & & \mathrm{B} & & & \mathrm{OH} & & \mathrm{B} & & & \mathrm{OH} & \end{array}$$

où S représente une molécule de sucre : le désoxyribose et B l'une ou l'autre des bases puriques ou pyrimidiques : adénine et guanine d'une part, cytosine et thymine d'autre part.

Il n'entrera pas dans le cadre de cet exposé de discuter des données relatives à la composition chimique proprement dite de ces molécules comme par exemple la proportion relative des diverses bases qui entrent en jeu, ou même la nature exacte de celles-ci.

Nous allons surtout nous placer dans un domaine beaucoup plus général qui relève d'une discipline nouvelle que l'on pourrait appeler la morphologie macromoléculaire, et nous examinerons le problème suivant.

Supposons que par une méthode donnée l'on ait extrait les acides nucléiques d'un milieu donné, nous nous proposons, en dissolvant l'échantillon dans un liquide approprié, de déterminer les proportions, les masses et les dimensions des diverses espèces de particules qui se trouvent dispersées dans le solvant.

Il s'agit donc du problème de l'analyse immédiate d'un milieu macromoléculaire, problème qui se pose d'une façon tout à fait générale aussi bien dans la chimie des hauts polymères que dans divers domaines de la biologie.

L'intérêt de la solution d'un tel problème, notamment dans le cas qui nous occupe, est assez clair si l'on admet que la méthode d'extraction employée est telle que l'on retrouve dans l'échantillon en expérience les espèces moléculaires qui existaient primitivement dans le milieu originel ; autrement dit si l'on admet qu'aucune dégradation ne se produit au cours des opérations. On comprend alors que l'analyse de l'échantillon *in vitro* puisse conduire à des conclusions importantes pour le biologiste et que les résultats obtenus puissent contribuer dans une mesure non négligeable à la détermination des caractères physico-chimiques liés à diverses spécificités biologiques, ce qui est un problème central de la physicochimie biologique.

Malheureusement, nous verrons qu'il n'en est pas encore ainsi. Au cours de l'extraction des actions diverses et encore mal identifiées se produisent, qu'il s'agisse d'actions mécaniques lors du broyage des tissus, ou de lyses plus ou moins poussées d'origine enzymatique ou strictement chimique, de sorte que l'échantillon préparé doit être considéré comme constitué très probablement par des débris des macromolécules originelles, avec des masses, ou des dimensions et des configurations géométriques qui varient d'un type de préparation à un autre.

Il est dès lors légitime de se demander si l'analyse morphologique d'un tel mélange présente bien un intérêt quelconque. Si l'on admet que les édifices macromoléculaires contenus dans le noyau ont été plus ou moins sévèrement endommagés au moment où on les a dégagés de leur gangue protéique, comment pourra-t-on, en faisant l'inventaire du tas de ruines que représente l'échantillon extrait, reconstituer l'architecture initiale qui, elle, est significative pour notre compréhension des phénomènes dont le noyau est le siège ?

Formulée d'une manière aussi absolue, la question est évidemment décourageante. Il est cependant nécessaire de la poser, de façon à éviter les illusions. Mais par ailleurs nous allons voir, dans ce qui suit, que les résultats très modestes, auxquels les physico-chimistes sont arrivés récemment, apportent une contribution non négligeable à l'effort mené en commun par les chimistes et par les biologistes.

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(*) Un certain nombre de résultats cités dans cet article ont été obtenus au C. R. M. par MM. J. Pouyet et G. Weill, que je remercie cordialement, ainsi que M. R. Vendrely, Chef du Service Biologique du C. R. M.

## DESCRIPTION SOMMAIRE DES MÉTHODES EMPLOYÉES

Il n'est peut-être pas inutile d'énumérer rapidement les types des méthodes de base communément employées, avec la signification des résultats qu'elles fournissent (1, 2).

C'est ce que nous allons faire en supposant tout d'abord que la solution en expérience contient un seul type de molécules (milieu monodisperse).

### *Solutions monodisperses*

*Diffusion de la lumière.* — C'est la méthode jusqu'ici la plus importante (3). Elle consiste à mesurer la distribution de l'intensité lumineuse diffractée dans l'espace par un élément de volume $\delta v$ de la solution de concentration $c$ (g./ml.).

Si cette concentration est suffisamment petite, on peut poser :

$$V_v = I_0 \frac{\delta v}{r^2} \cdot \frac{4\pi^2}{\lambda^4} \cdot n_0^2 \left(\frac{n - n_0}{c}\right)^2 \cdot c\frac{M}{N_0} \cdot P_v(\theta) \qquad (1)$$

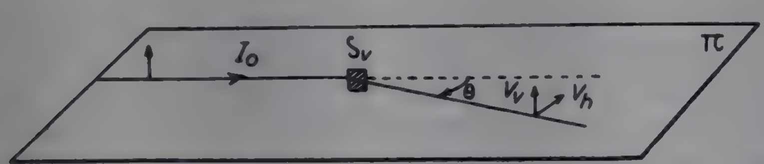


Fig. 1. — Les symboles sont identiques à ceux utilisés pour l'équation *1*.

Dans cette expression :

- $I_0$ représente l'intensité du faisceau de lumière parallèle incident de longueur d'onde $\lambda$, que nous supposerons polarisé perpendiculairement au plan Π (figure 1),
- $V_v$ est l'intensité du faisceau polarisé verticalement diffusé dans la direction $\theta$ et observé à la distance $r$ de $\delta v$,
- $n_0$ est l'indice du solvant pur,
- $n$ celui de la solution,
- $M$ la masse moléculaire des macromolécules en solution,
- $N_0$ le nombre d'Avogadro,
- $P_v(\theta)$ une fonction de $\theta$, sans dimensions, et plus petite que l'unité.

On voit que l'on peut écrire cette équation sous la forme :

$$\frac{c}{K} = \frac{1}{M} P_v^{-1}(\theta)$$

où $K$ représente un facteur accessible à la mesure.

En réalité, la formule précédente peut être remplacee par l'équation plus générale :

$$\frac{c}{K} = \left(\frac{1}{M} + 2\,Bc\right) P_v^{-1}(\theta) \qquad (2)$$

où $B$ représente le deuxième coefficient de viriel de la solution.

En général, il peut arriver qu'il existe une intensité non nulle diffusée correspondant à une composante $V_h$ (polarisée horizontalement), ce qui amène une complication sérieuse à la théorie.

Nous laissons ce cas de côté puisque, dans les solutions d'acide nucléique on observe que $V_h = 0$.

Dans ce cas il est possible de calculer complètement la fonction $P_v(\theta)$ dans le cas où les macromolécules qui diffusent sont des bâtonntes très allongés, des sphères, ou des filaments pelotonnés d'une façon statistique selon ce qu'on appelle des pelotes de Gauss.

Dans le cas général, quelle que soit la forme de la macromolécule, on démontre que les deux premiers termes du développement de $P_v^{-1}(\theta)$ en fonction de $\theta$ sont donnés par :

$$P_v^{-1}(\theta) = 1 + \frac{\mu^2 r_g^2}{3} + \ldots \qquad (3)$$

où :

$$\mu = \frac{4\pi n_0}{\lambda} \sin\frac{\theta}{2} \qquad (4)$$

et où $r_g$ est le rayon de giration de la macromolécule, c'est-à-dire la grandeur définie par :

$$r_g^2 = \frac{1}{N} \sum_{i=0}^{N} r_i^2 \qquad (5)$$

Dans cette équation, $r_i$ représente la distance de chacun des $N$ points $A_i$ de la molécule au centre de gravité de celle-ci.

La grandeur dépend de la forme et des dimensions de la molécule. Par exemple, voici les valeurs de $r_g^2$ dans les cas simples suivants :

$r_g^2 = L^2/12$ pour un bâtonnet de longueur $L$,
$r_g^2 = 3\,L^2/20$ pour une sphère de diamètre $L$, (6)
$r_g^2 = L^2/6$ pour une pelote de Gauss à $N$ éléments statistiques de longueur $b$ ($L^2 = Nb^2$).

Enfin, il faut remarquer que l'équation (*2*) n'est valable que pour les valeurs de $c$ voisines de zéro.

L'expérience consistera donc à mesurer $\frac{c}{K}$, $\theta$ étant fixe, pour des valeurs de $c$ tendant vers zéro; puis à faire la même opération pour des valeurs différentes de $\theta$ de plus en plus voisines de zéro. On obtient ainsi 2 courbes extrapolées.

L'une donne les valeurs de $\left(\frac{c}{K}\right)$ en fonction de $c$ pour $\theta = 0$, valeur pour laquelle $P_v^{-1}(\theta) = 1$. Cette courbe a donc pour équation :

$$\left(\frac{c}{K}\right)_{\theta=0} = \frac{1}{M} + 2\,Bc \qquad \text{(figure 2a)} \qquad (7)$$

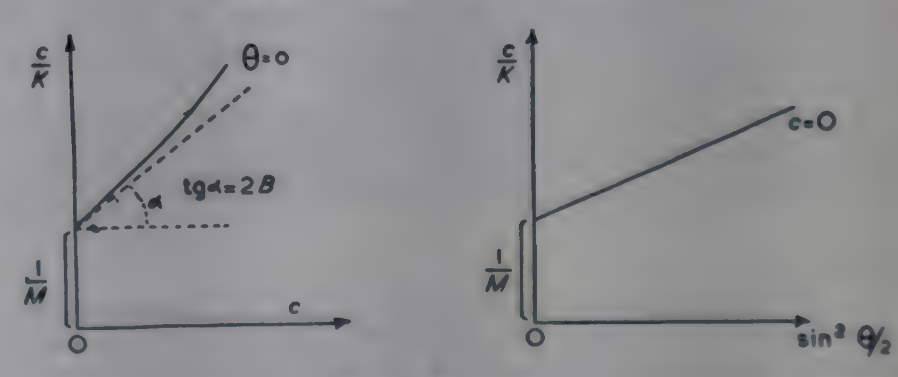


Fig. 2*a* et 2*b*.

Le point où elle coupe l'axe des ordonnées donne $\frac{1}{M}$ et sa pente à l'origine donne $B$.

L'autre donne $\frac{c}{K}$ en fonction de $\theta$ pour $c = 0$. Son équation est :

$$\left(\frac{c}{K}\right)_{c=0} = \frac{1}{M}\left(1 + \frac{\mu^2 r g^2}{3}\right) \quad \text{(fig. 2}b\text{)} \qquad (8)$$

Le point où elle coupe l'axe des ordonnées donne $\frac{1}{M}$ et sa pente permet de calculer $r_g^2$ puisque $\mu^2$ est connu (équation *4*).

En résumé la diffusion de la lumière donne $M$, $B$ et le rayon de giration de la particule.

On peut également faire le rapport entre les intensités diffusées par la solution entre deux angles $\theta$ tels que 45° et 135°. On obtient alors d'après (*1*) :

$$\frac{V_v\,(45^\circ)}{V_v\,(135^\circ)} = \frac{P_v\,(45^\circ)}{P_v\,(135^\circ)} = \delta_s \qquad (9)$$

Le nombre $\delta_s$ ne dépend que de la forme et des dimensions des particules. On peut calculer entièrement sa valeur par la théorie dans le cas des bâtonnets, sphères et pelotes de Gauss. On a reporté les résultats dans le graphique de la figure.

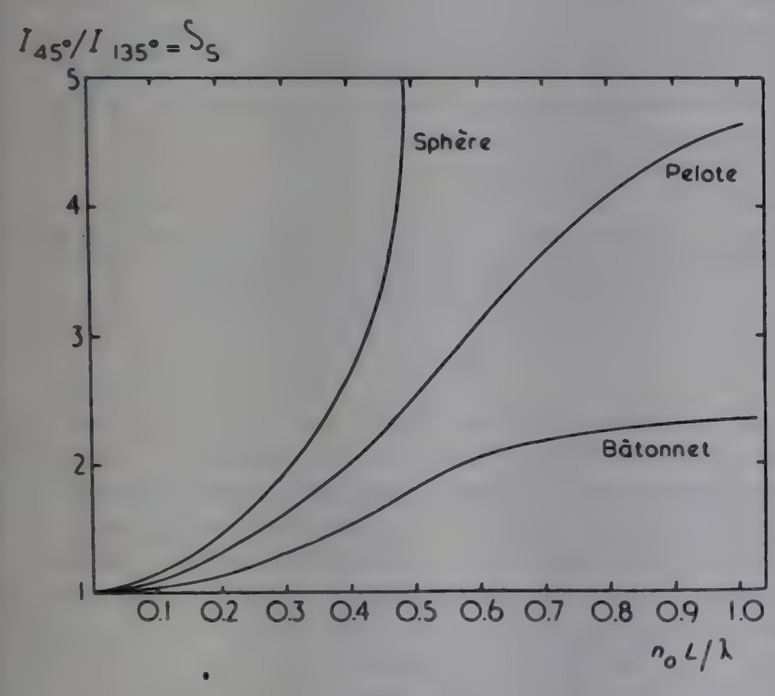


Fig. 3.

La mesure de $\delta_s$, qui a l'avantage d'être rapide et de ne pas nécessiter l'étalonnage préalable de l'appareil, permet donc de déterminer la dimension du bâtonnet de la sphère ou de la pelote pour peu qu'on sache par avance à quel type de molécules l'on a affaire. Remarquons que la valeur de $\delta_s$, pour un bâtonnet, ne peut dépasser une limite égale à 2.4.

*Ultracentrifugation* (*vitesse de sédimentation*) — Cette méthode est trop connue des biologistes pour qu'il soit nécessaire d'y insister. On sait qu'elle consiste en la mesure d'une grandeur $S$, la constante de sédimentation, dont l'expression est :

$$S = \frac{M(1 - \overline{V}\rho)}{N_0 f} \qquad (10)$$

où $M$ est la masse moléculaire et $N_0$ le nombre d'Avogadro comme plus haut ; où $\rho$ est la densité du solvant à la température de l'expérience ; où $f$ est le frottement éprouvé par la particule pour la vitesse unité, dans son déplacement uniforme au cours de la sédimentation, et où $\overline{V}$ est le volume spécifique de la particule.

On voit que la mesure de la vitesse de sédimentation donne une grandeur dépendant à la fois de la masse et, par l'intermédiaire de $f$, de la forme et dimension de la macromolécule.

L'équation (*10*) n'est strictement valable, à cause des interactions entre macromolécules, que pour une concentration nulle. D'où encore la nécessité d'une extrapolation pour $c = 0$ des résultats de mesure.

*Viscosité.* — Si $\eta$ est le coefficient de viscosité de la solution, $\eta_0$ celui du solvant pur, le rapport :

$$\frac{\eta - \eta_0}{\eta_0}$$

appelé viscosité spécifique est, à condition que la concentration $c$ soit assez faible pour que les interactions entre macromolécules soient négligeables, proportionnel au nombre $N_0 \frac{c}{M}$ de celles-ci par ml. et le coeffiicent de proportionnalité dépend de la forme et de leurs dimensions.

On a donc :

$$[\eta] = \left(\frac{\eta - \eta_0}{\eta_0\, c}\right)_{c=0} = \frac{N_0\, F}{M}$$

On a coutume de désigner $[\eta]$ sous le nom de viscosité intrinsèque.

Le facteur $F$ peut être calculé théoriquement dans certains cas simples, en supposant valables à l'échelle qui nous intéresse les lois de l'hydrodynamique des fluides visqueux continus.

Dans le cas d'une particule de forme ellipsoïdale dont le volume d'encombrement (qui n'est pas nécessairement égal au volume propre de la molécule) est $V_e$, on a :

$$[\eta] = \frac{V_e\, N_0}{M} \Lambda(p) \qquad (11)$$

où $\Lambda(p)$ est une fonction croissante de l'allongement $p$ de la particule et dont la valeur minima est égale à 2.5 pour $p = 1$, c'est-à-dire pour la sphère.

Par exemple, pour un bâtonnet très allongé, on a approximativement :

$$\Lambda(p) = \frac{4\,p^2}{15} \frac{1}{\ln 2\,p}$$

c'est-à-dire que $\Lambda(p)$ est sensiblement proportionnel au carré de la longueur de la particule, ou au carré de son rayon de giration.

Dans le cas d'une particule en pelote, il est plus difficile de donner une expression valable de la viscosité intrin-

sèque, mais de l'ensemble de nos connaissances expérimentales sur les hauts polymères on peut proposer une loi de la forme :

$$[\eta] = \varphi \frac{N_0}{M} (\overline{R^2})^{3/2} \qquad (12)$$

où $R^2$ est le carré moyen de la distance entre les extrémités de la pelote, et $\varphi$ un coefficient dont la valeur (4) est telle que $\varphi N_0 = 2.1 \times 10^{23}$.

En tout cas $[\eta]$ étant proportionnel à $(\overline{R^2})^{3/2}$ est de ce fait proportionnel au cube du rayon de giration.

Ce qu'il convient de remarquer, c'est que si l'on considère une particule de masse donnée $M$, la valeur de $[\eta]$ est la plus grande quand la particule a la forme d'un bâtonnet très allongé, qu'elle est plus petite si le bâtonnet est coudé en pelote de Gauss, et qu'enfin elle est minima si la particule est une sphère compacte.

*Exemple* : Considérons des ordres de grandeur comparables à ceux que l'on trouve dans l'étude des solutions d'ADN et prenons : $M = 4 \times 10^6, \frac{V_e}{M} = 0.94$.

Bâtonnet : $L = 20\,000$ Å, diamètre 20 Å ; on trouve $[\eta] = 24\,500$.

Sphère compacte : $[\eta] = 2.35$.

Pelote de Gauss : avec N = 100 éléments statistiques de longueur $b = 200$ Å ; $[\eta] = 420$.

Enfin il convient de faire une remarque d'une grande importance dans le cas où les particules en solution sont de grandes dimensions et fortement anisodiamétriques. Au cours de l'écoulement dans le viscosimètre de telles particules, à cause de leur faible mouvement brownien de rotation, subissent une orientation non négligeable dans la direction du flux et d'autant plus importante que les gradients de vitesse sont élevés. On constate alors que le coefficient de viscosité mesuré dépend de la vitesse d'écoulement.

Il est alors nécessaire, si l'on veut interpréter simplement la viscosité intrinsèque à partir de la théorie et utiliser les équations telles que (*11*) par exemple, de procéder à une extrapolation des mesures à un gradient de vitesse nulle.

Nous en verrons un exemple plus loin.

Dans ce cas, l'emploi d'un viscosimètre du type Couette est à conseiller.

*Autres méthodes.* — L'étude de la diffusion brownienne libre, de la biréfringence d'écoulement aussi bien que celle de l'effet Kerr (5) ou de la diffusion de la lumière dans un champ d'orientation donne des renseignements importants.

Comme, dans le cas présent, nous ne signalerons les résultats donnés par ces méthodes qu'à titre épisodique, nous n'alourdirons pas cet exposé en en discutant les principes ni les résultats.

### *Solutions polydisperses*

L'emploi systématique des méthodes que nous venons d'énumérer, la comparaison et la discussion des résultats qu'elles fournissent permet, dans le cas où la solution contient une seule espèce de particules, d'obtenir des résultats précieux sur la masse, la dimension et la forme de celles-ci.

Il est aussi possible de choisir entre diverses structures proposées par le chimiste et le biologiste, et même de suivre certaines modifications structurales produites par certains agents extérieurs (par ex. température, pH, force ionique du milieu).

Mais si, par contre, la solution contient un mélange d'espèces différentes, l'on conçoit que ces mêmes méthodes fournissent des valeurs moyennes des masses, formes et dimensions et qu'il devient alors extrêmement compliqué de tirer de leur emploi autre chose que des résultats souvent grossièrement qualitatifs.

Seule la mesure directe des masses conduit à un résultat quantitatif interprétable. Par exemple la diffusion de la lumière fournit la moyenne en poids $\overline{M}_p$ des masses moléculaires définie par :

$$\overline{M}_p = \int_0^\infty M^2 f(M)\, dM \Big/ \int_0^\infty M f(M)\, dM = \int_0^\infty Mg(M)\, dM \Big/ \int_0^\infty g(M)\, dM \qquad (13)$$

où $f(M)dM$ représente le pourcentage du nombre et $g(M)dM$ le pourcentage en poids de molécules dissoutes et dont la masse est comprise entre $M$ et $M + dM$.

En ce qui concerne les dimensions, l'établissement de moyennes définies, par exemple pour le rayon de giration, n'est possible que si toutes les particules sont géométriquement semblables et ne diffèrent que par la valeur d'un seul paramètre caractéristique. Nous laisserons de côté ce cas particulier, et nous noterons, en conclusion qu'il faut s'attendre à ce que, dans une solution polydisperse, l'analyse morphologique ne conduise qu'à des résultats qualitatifs et incertains.

## APPLICATION AUX SOLUTIONS D'ACIDE DESOXYRIBONUCLEIQUE

### *Le solvant*

Les échantillons d'acide désoxyribonucléique préparés par les méthodes habituelles sont solubles dans l'eau ainsi que dans les solutions chlorurées sodiques en toutes concentrations de NaCl. L'emploi de l'eau pure ou de la solution chlorurée n'est cependant pas indifférent.

En effet, nous avons indiqué ci-dessus que l'emploi correct des méthodes d'analyse physico-chimique supposait que les résultats de mesure fussent extrapolés à concentration nulle, et cela afin d'éviter entre les particules des interactions dont l'effet peut être beaucoup plus grand que celui du phénomène que l'on peut interpréter théoriquement.

Or d'une part les molécules de ADN sont très encombrantes et, d'autre part, elles portent en solution des charges électriques négatives qui proviennent de l'ionisation du sel de sodium.

Il en résulte, entre molécules voisines en solution aqueuse des interactions intenses qui se marquent par un taux considérable de variation des phénomènes mesurés en fonction de la concentration en ADN. Il est alors nécessaire d'opérer à de très hautes dilutions si l'on veut avoir une précision convenable pour les valeurs

extrapolées à concentration nulle, ce qui présente de réelles difficultés techniques.

La situation est considérablement améliorée si on opère en solution saline : chlorurée sodique par exemple. Alors les charges libres sont pratiquement saturées et les interactions coulombiennes disparaissent.

Les résultats de mesure (6, 7) sur la viscosité des solutions d'un échantillon SVIII (voir plus loin), portés sur la figure 4 illustrent bien ces conclusions.

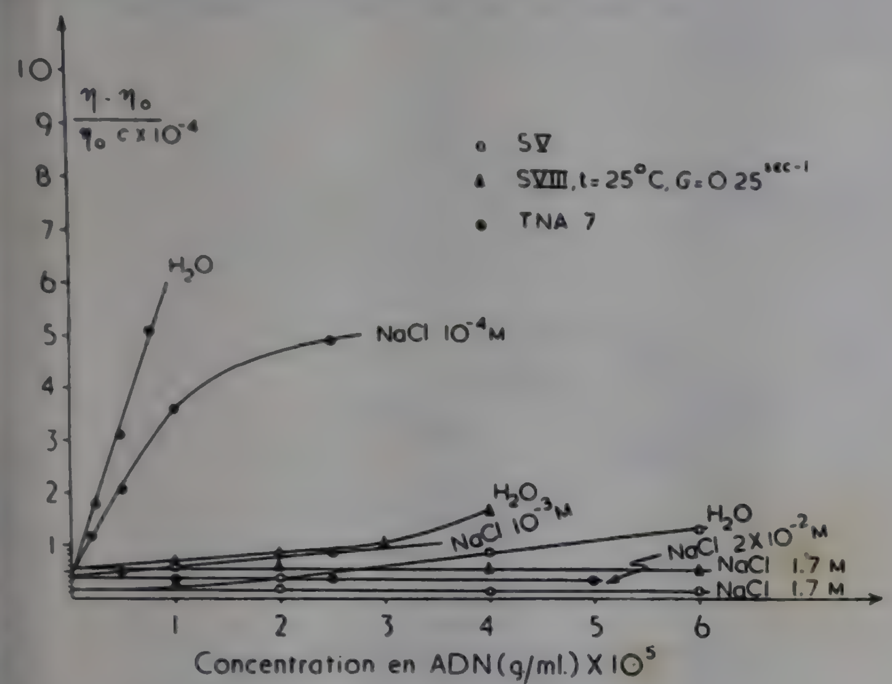


FIG. 4. — Valeurs de $\frac{\eta' - \eta_0}{\eta_0 c}$ en fonction de la concentration en ADN pour divers échantillons, en solution aqueuse ou chlorurée (6, 7).

La situation est la même dans le cas de la diffusion de la lumière par les solutions aqueuses, où les extrapolations donnent des résultats pratiquement illusoires.

Sans vouloir discuter encore ce point en détail, il est cependant nécessaire de remarquer que la présence du chlorure de sodium n'altère ni la masse ni les dimensions des molécules d'ADN puisque la valeur de la viscosité intrinsèque, obtenue par extrapolation, est la même dans la limite de la précision des mesures.

Il s'ensuit que l'emploi des solutions chlorurées est, dans ce cas, d'un intérêt particulier.

Remarquons en même temps que, sur cet exemple, les concentrations en ADN employées sont très basses : de $2 \times 10^{-5}$ à $10^{-4}$ g./ml. L'expérience montre d'une manière tout à fait générale qu'il faut opérer dans ce domaine de dilution si l'on veut obtenir des résultats susceptibles d'être extrapolés avec une certaine sûreté.

### *Les masses*

L'expérience montre que la valeur de la moyenne $\overline{M}_p$ déterminée par la diffusion de la lumière dépend beaucoup du type de préparation employé ainsi que des opérateurs, même s'il s'agit du même tissu d'origine, qui est toujours, dans les expériences citées, le thymus du veau.

Nous avons rassemblé un certain nombre de données récentes dans le tableau ci-dessous.

De la dispersion des résultats obtenus nous pouvons tirer quelques conclusions.

— Puisque la masse des particules obtenues dépend du mode d'extraction, il est raisonnable de penser que chaque préparation contient un mélange de particules de masses différentes et que les solutions sont polydisperses. Une preuve directe de ce fait est d'ailleurs observable dans les résultats obtenus sur l'effet Kerr présenté par des solutions aqueuses de SVIII et SV (5).

— Pour la même raison, si l'on suppose que dans le noyau de la cellule il existe effectivement des molécules bien définies d'ADN, les molécules dispersées dans la solution ne sont pas celles qui existaient *in situ*. En dégageant celles-ci de leur environnement protéique, on en a modifié la masse.

Deux hypothèses sont alors permises : où bien, au cours des opérations d'extraction, les molécules originelles ont été soudées les unes aux autres en nombre plus ou moins grand, par exemple par des ponts d'hydrogène ou bien, ce qui semble plus probable, les molécules originelles ont

TABLEAU I

| Type | Numéro de la préparation | Masse × $10^{-6}$ | Auteurs | $r_g$ en Å | $[\eta]$ |
|---|---|---|---|---|---|
| C. V. 3 | 1 (réf. 10) | 1.9 | C. R. M. | 1100 | 850 |
| B. G. | 8 (réf. 17 et 18) | 3.53 | Doty | 1630 | 3070 |
| S. X | 4 (réf. 15) | 3.85 | C. R. M. | 1460 | 1500 |
| Dounce | 9 (réf. 19, 20 et 21) | 4.69 | Doty | 1830 | — |
| Chargaff | 9 (réf. 19, 20 et 21) | 4.69 | Doty | 2036 | — |
| Simmons | 10 (réf. 22 et 23) | 5.86 | Doty | 2020 | 5340 |
| S VII | 3 (réf. 13 et 14) | 5.92 | Doty | 2200 | 5100 |
| S V | 3 (réf. 13 et 14) | 6.0 | C. R. M. | 2036 | 1350 |
| S VIII | 3 (réf. 13 et 14) | 6.0 | C. R. M. | 2660 | 5300 |
| Varin | 8 (réf. 17 et 18) | 6.87 | Doty | 2090 | 4800 |
| Varin (Signer) | 3 (réf. 13 et 14) | 7.7 | Doty | — | 6000 |
| Doty-Price | 10 (réf. 22 et 23) | 7.7 | Doty | 2900 | 7200 |
| TNA7 | 7 (réf. 24) | 8.5 | C. R. M. | 2400 | 4000 |
| S XII | 4 (réf. 15) | 11.6 | C. R. M. | 2240 | 3650 |
| C V 9 | 5 (réf. 16) | 15 | C. R. M. | 2100 | 4000 |
| C V 5 | 6 (réf. 16) | 16.5 | C. R. M. | 2550 | 3000 |

été dégradées de manière variable, par exemple par action mécanique ou enzymatique.

Si l'on admet cette deuxième hypothèse, il semble logique de conclure que, plus la masse obtenue est élevée, plus la méthode d'extraction a respecté la masse des particules originelles.

Dans ce cas, la mesure des masses fournit un guide pour le biochimiste dans ses opérations préparatives.

Si donc, malgré le perfectionnement systématique des conditions de préparation (précautions dans le broyage, blocage des enzymes, etc...), on n'arrive pas à dépasser une grandeur limite de la masse, il est permis d'espérer que l'on est arrivé à une extraction parfaite, ne donnant pas de dégradation appréciable.

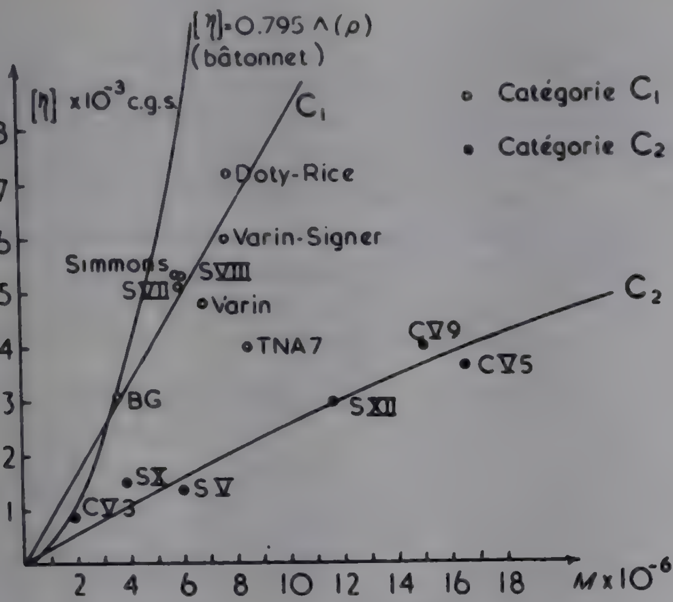


FIG. 5. — $[\eta] = f(M)$.

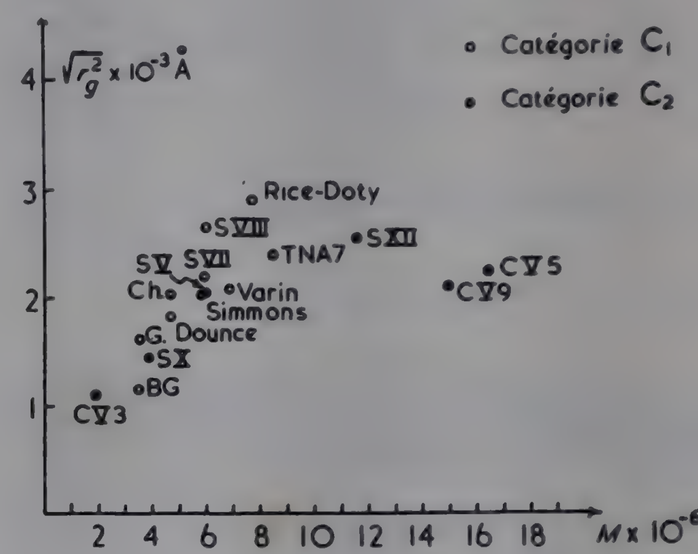


FIG. 6. — $\sqrt{r_g^2} = f(M)$.

Cela ne veut pas dire, évidemment, que la solution correspondante est nécessairement monodisperse, rien n'indiquant à priori que les molécules originelles d'ADN aient la même masse.

— De ce point de vue, il apparaît, d'après le tableau I, que l'échantillon du type C V 5 dont la masse moyenne est $\overline{M}_p = 16.5 \times 10^6$ serait le meilleur.

On peut en tout cas observer que si l'on admet que cette masse a une signification il y correspondrait, d'après le modèle bien connu proposé par Crick et Watson et d'après lequel la molécule d'ADN serait un filament en double spirale dont la masse molaire est de 20 000 par 100 Å, une longueur totale moléculaire de 8.25 microns.

Cette longueur des éléments est énorme par rapport aux éléments biologiques originels et l'on doit admettre par conséquent que la molécule originelle d'ADN ne peut avoir, *in situ*, la configuration linéaire ou quasi linéaire observée au microscope électronique.

Il est nécessaire de supposer, au contraire, qu'elle est fortement plissée ou repliée sur elle-même. Il faut donc qu'il existe, le long du filament, des articulations plus ou moins libres permettant ce plissement.

### *Les configurations et dimensions*

*Les deux catégories d'échantillons.* — Puisque les solutions qui se présentent à l'examen contiennent, à l'exception peut-être d'un seul cas, des débris divers des macromolécules natives, il semble bien que les interprétations quantitatives tirées d'une étude poussée, outre qu'elles soient difficilement accessibles, ne présentent pas une grande signification. Par contre les interprétations qualitatives semblent plus sûres et également plus intéressantes dans l'état actuel des choses. La première constatation de cet ordre et qui apparaît avec évidence est la suivante. Considérons les valeurs de la viscosité intrinsèque et du rayon de giration des divers échantillons (tableau I) et portons-les sur un graphique en fonction des masses (figures 5 et 6).

Un simple examen montre que les points représentant les valeurs de $[\eta]$ se placent, en gros, sur deux courbes différentes et que l'on peut classer les échantillons en deux catégories $C_1$ et $C_2$, à l'exception de l'échantillon C V 3 qui, en raison de sa faible masse, peut appartenir tout aussi bien à l'une qu'à l'autre, et l'échantillon T N A 7 (*) qui est nettement intermédiaire. On observe la même différence mais moins nette avec les rayons de giration, ceux de la catégorie $C_1$ étant supérieurs à ceux de la catégorie $C_2$.

Pour l'instant nous n'avons aucune indication sur des différences typiques entre les méthodes d'extraction à quoi seraient dues ces différences de propriétés.

Avant de rechercher quelles peuvent être les différences de structure entre les molécules appartenant aux deux catégories, signalons quelques propriétés communes.

*Type général des molécules d'acide nucléique.* — La première de celles-ci, et que nous avons déjà signalée, c'est que la viscosité intrinsèque est, ainsi que cela est apparu sur les échantillons S V et S VIII, qui appartiennent à des catégories différentes, indépendante de la présence dans la solution d'un électrolyte tel que NaCl.

Cela indique que les configurations sont indépendantes des répulsions qu'exercent entre elles les charges portées par la molécule ionisée dans la solution aqueuse.

Donc les configurations macromoléculaires, quelles qu'elles soient, sont rigides et les molécules d'ADN, en

(*) La protéine a été extraite par une méthode différente dans son principe (digestion enzymatique).

solution aqueuse, ne sont pas comparables à des molécules de polyélectrolyte en chaîne flexible. D'ailleurs, l'allure générale des courbes donnant $(\eta - \eta_0)/\eta_0 c$ en fonction de $c$ est différente dans les deux cas, comme le montre la figure 7, relative à un polyélectrolyte en chaîne (25), et que l'on composera à la figure 4 relative à S V et S VIII.

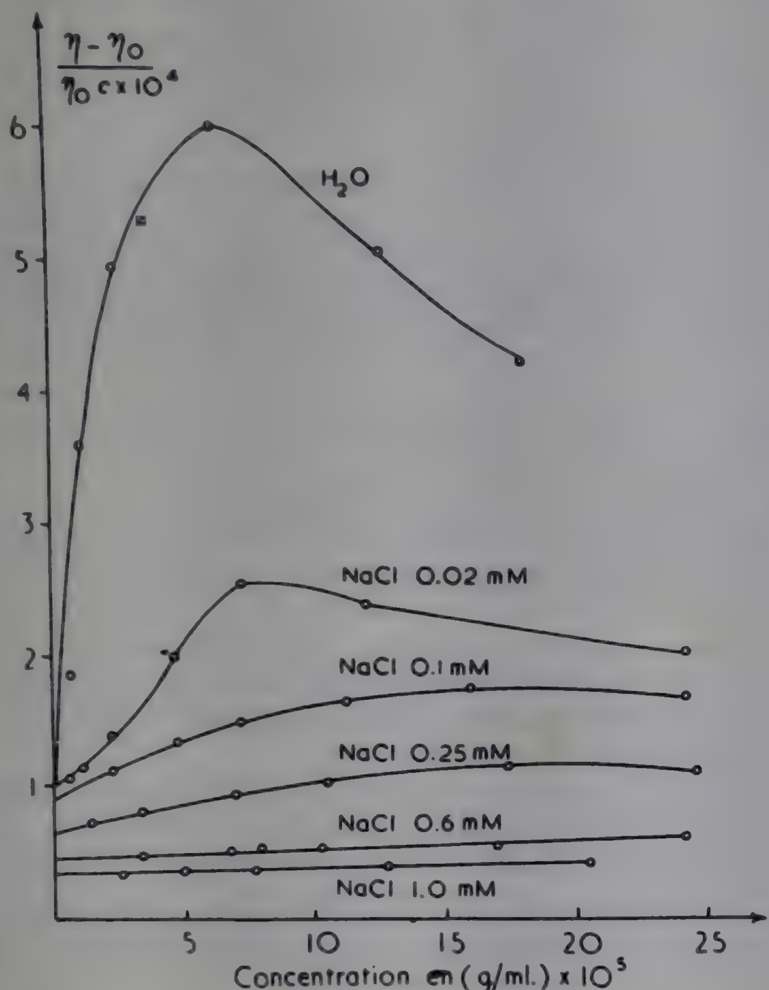


FIG. 7. — Valeurs de $\frac{\eta - \eta_0}{\eta_0 c}$ des solutions aqueuses et salines de bromure de polyvinylbutylpyridinium (25). $t = 25°$ C.; $G = 0.25$ cm$^{-1}$.

Si les macromolécules d'ADN sont rigides, elles ne peuvent pas être de simples bâtonnets.

La raison primordiale en est que le coefficient de dissymétrie $\delta_s$ observé par diffusion de la lumière, qui dépend de la masse, est toujours supérieur à la valeur limite 2.4 imposée par la théorie pour les bâtonnets de longueur infinie. Par exemple, pour S V et S VIII, $\delta_s$ est égal à 3.8.

D'ailleurs si l'on admet une structure du type de celle proposée par Crick et Watson, nous avons vu que la longueur obtenue pour la molécule rigide et rectiligne n'est pas acceptable.

Enfin, dans cette même hypothèse, on peut calculer la viscosité intrinsèque d'une solution monodisperse dont l'expression est de la forme (voir équation *11*).

$$[\eta] = A \, \Lambda \, (p).$$

Nous avons calculé $A$ en supposant que l'échantillon B.G était constitué par un bâtonnet rigide correspondant à la double spirale de Crick et Watson et nous avons reporté l'expression obtenue sur la figure 5 (trait discontinu).

On voit que pour des masses supérieures à $5 \times 10^6$ la viscosité calculée est hors de proportion avec les résultats de mesure.

Ces conclusions seraient encore aggravées dans l'hypothèse où la molécule de ADN serait formée non de deux chaînes soudées, comme dans le cas de la double spirale, mais d'une seule.

Il est donc indispensable de supposer que le filament formé par la chaîne de ADN, couplée ou non avec une chaîne semblable, soit coudé ou replié sur lui-même de façon régulière ou non, comme on pourrait le faire avec un fil de fer rigide.

Toujours est-il que les lignes brisées ainsi dessinées par les diverses espèces macromoléculaires contenues dans la solution polydisperse ne peuvent avoir toutes une symétrie sphérique. Sinon, en effet on ne pourrait expliquer, la déformation étant exclue du fait de la rigidité, que l'on observe une biréfringence d'écoulement de la solution.

*Comparaison entre les molécules des catégories $C_1$ et $C_2$.* — Les molécules des deux catégories, nous venons de le voir, correspondent au même modèle général. Qu'il s'agisse de $C_1$ ou de $C_2$ les solutions contiendraient des molécules en ligne brisée de masses, donc de longueurs totales, différentes et probablement d'allongements différents.

Dès lors, les différences observées sur les deux catégories s'expliquent très simplement en admettant que la proportion des configurations allongées est plus grande pour $C_1$ que pour $C_2$.

Puisque (voir méthodes), la viscosité est beaucoup plus grande pour un allongement élevé (à la limite un bâtonnet) que pour une pelote (à la limite pelote de Gauss), il suffit, pour expliquer l'effet observé de supposer que $C_1$ contienne des configurations allongées en proportion à peine plus grande que pour $C_2$.

En même temps s'expliquerait aussi le fait que les rayons de giration de $C_1$ sont plus élevés que pour $C_2$ (figure 6), mais cependant que l'augmentation observée soit d'un taux moins élevé qu'en ce qui concerne la viscosité.

Ce fait s'explique d'ailleurs aisément puisque il est proportionnel au cube du rayon de giration.

Du même coup également s'expliquent deux observations que nous n'avons pas encore signalées.

La première est relative à l'effet du gradient de vitesse $G$ sur la viscosité (8). On observe (figure 8) que cet effet est beaucoup plus grand pour S VIII et pour S V, alors que la masse moyenne de ces deux échantillons est la même. Cela indique que, en moyenne, l'orientation des particules est plus grande pour le premier que pour le deuxième, donc que dans le premier cas, l'allongement des particules est en moyenne plus grand que dans le deuxième.

La deuxième est relative à la biréfringence d'écoulement (9) pour ces mêmes substances (figure 9) en fonction du gradient $G$.

La position relative des isoclines $\Psi(G)$ et des courbes de biréfringence $\Delta n$ montrent sans conteste que le taux d'orientation, pour un même gradient est plus grand pour S VIII que pour S V.

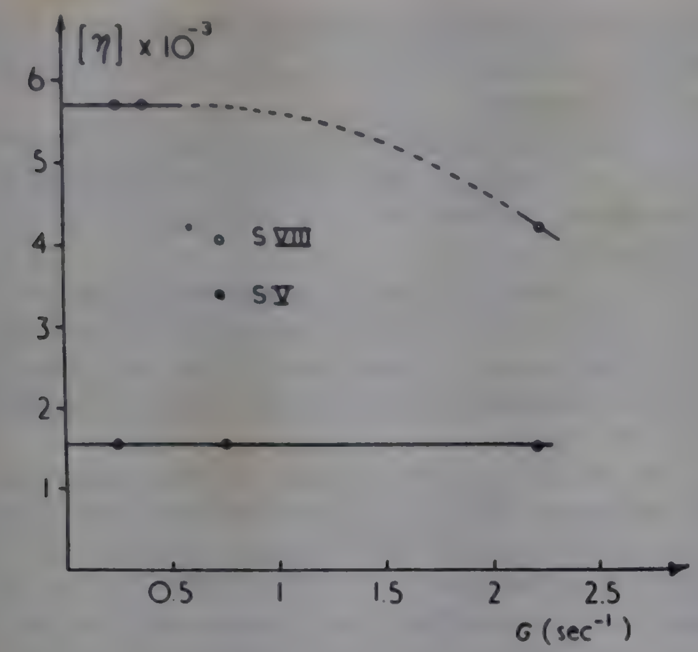


FIG. 8. — $t = 25°$ C. ; $c$ NaCl = .1.7 M.

Remarquons que, ci-dessus, nous avons évité toute précision sur la statistique des configurations des particules car, dans une solution polydisperse, où l'on ignore la distribution de leurs masses, il est impossible de donner une interprétation quantitative des résultats de mesure.

On peut tout de même se hasarder à une hypothèse qui n'est pas déraisonnable, en même temps qu'elle est d'une grande généralité. Elle consiste à supposer que la direction d'un élément de la ligne brisée schématisant la macromolécule est indépendante de la direction des éléments voisins. Si donc $N$ représente le nombre de segments dont les longueurs ont pour carré moyen $b^2$ on a affaire à une « pelote de Gauss », l'un des cas classiques envisagé dans la deuxième partie de cet exposé.

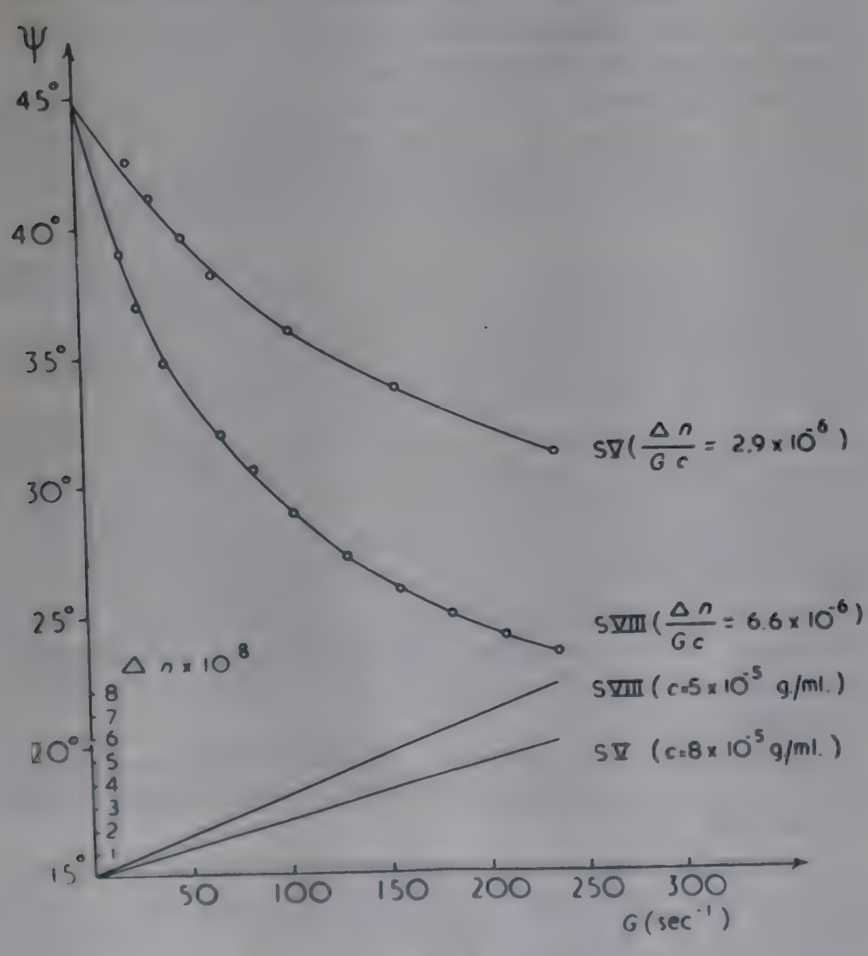


FIG. 9.

Dans ce cas, on peut interpréter les résultats observés en supposant que les solutions contiennent des pelotes dont la longueur linéaire $L = Nb$, proportionnelle à la masse M, varie de l'une à l'autre.

En effet, une répartition de Gauss des configurations des particules rigides comprend des configurations anisodiamétriques responsables des effets d'orientation dans les champs hydrodynamiques observés dans la mesure de la viscosité ou dans celle de la biréfringence d'écoulement.

L'interprétation des différences observées entre les catégories $C_1$ et $C_2$ peut alors être tentée sur la base de la structure en pelote.

Par exemple, on peut supposer que les pelotes de type $C_1$ contiennent moins de coudes par unité de longueur que celles du type $C_2$ et que, par conséquent, la valeur de $b$ dans le premier cas est plus élevée que dans le deuxième. Mais bien d'autres hypothèses sont également possibles.

*Action de la chaleur sur les particules d'ADN en solution.* — On a consacré beaucoup d'efforts à l'action de divers agents sur les molécules d'ADN.

Il sortirait du cadre de cet exposé d'en rendre compte de façon complète et nous nous bornerons à l'étude de l'effet d'une augmentation de température sur les solutions.

Il faut d'abord remarquer que, bien souvent, il est difficile de tirer des conclusions sûres des observations qui ont été faites dans des conditions expérimentales généralement trop mal définies.

En effet, il semble qu'en général trois effets puissent se produire soit séparément, soit simultanément, selon l'intensité ou la durée de l'action exercée. Ce sont :

— Un changement de configuration des macromolécules sans changement de masse ni de constitution (par exemple passage d'un bâtonnet à une pelote).

— Un changement de masse sans changement de constitution (par exemple coupure du filament en tronçons plus petits). Chaque morceau est alors, aux dimensions près, de même constitution que la macromolécule initiale.

— Un changement radical de l'édifice moléculaire produit par une véritable dissociation d'un très grand nombre de liaisons internes et faisant apparaître des débris dont la constitution est différente de celle de la molécule initiale.

Comme les propriétés générales des solutions, telles que nous les avons exposées plus haut, sont hautement affectées par chacun des trois phénomènes, on conçoit la difficulté de l'interprétation d'une expérience qui n'est pas menée rigoureusement et de façon quantitative, ainsi que les confusions qui peuvent résulter d'une observation superficielle et qualitative.

Cela posé, nous examinerons très brièvement quelques résultats acquis tels qu'ils apparaissent dans deux récents mémoires (26, 27). Nous y joindrons quelques récents compléments obtenus au cours des travaux qui sont effectués actuellement dans notre laboratoire, et qui sont en voie de développement.

Tout d'abord, on se trouve devant deux observations d'apparence contradictoire. Dans un cas, on trouve que le traitement thermique en solution saline neutre (26)

à 100° C. pendant 15 minutes produit une diminution de masse, dans l'autre (27) que la masse reste constante.

Nous avons observé que le traitement en question a donné les résultats suivants :

— Solution neutre aqueuse à 20 mg./100 ml. (masses et rayons de giration mesurés par diffusion de la lumière)

| | | |
|---|---|---|
| S V | $M = 280\,000$ | $r_g = 275$ Å |
| S VIII | $M = 350\,000$ | $r_g = 260$ Å |

— La même solution avec NaCl 0.2 M :

| | | |
|---|---|---|
| S V | $M = 2.4 \times 10^6$ | $r_g = 850$ Å |
| S VIII | $M = 1.5 \times 10^6$ | $r_g = 700$ Å |

Dans les deux cas, on n'observe pas de changement sensible de la densité optique dans la bande d'absorption ultra-violette de 260 mμ pendant la dégradation.

On constate ainsi qu'il y a sans aucun doute une diminution de la masse, dans les conditions opératoires réalisées, mais que la présence de NaCl retarde la dégradation.

D'autre part, l'absorption ultraviolette n'ayant pas changé, il est difficile de supposer que, ainsi que le suppose l'un des auteurs (26), cette dégradation soit accompagnée de la rupture d'un nombre important de liaisons hydrogènes.

On remarquera de plus que, dans la solution aqueuse, les produits de dégradation sont sensiblement identiques, qu'il s'agisse de S V ou de S VIII, ce qui semblerait indiquer que les deux structures sont constituées des mêmes éléments de base. Si on suppose que ceux-ci sont des bâtonnets, ce qui est compatible avec la valeur $\delta_s = 1.1$ du coefficient de dissymétrie, ceux-ci auraient une longueur moyenne de 900 Å ce qui, avec le modèle de la double spirale, conduit à une masse d'environ 200 000.

Il est difficile de dire si ce résultat est vraiment incompatible avec la valeur moyenne de 300 000 mesurée directement par diffusion de la lumière, étant donné la polydispersité du milieu.

Nous pensons qu'il est nécessaire d'attendre, avant de pousser plus loin l'interprétation des résultats, que des mesures systématiques aient été faites.

Elles nous montreront sans doute que les observations différentes relatées dans les deux mémoires cités proviennent d'une différence dans les conditions de l'expérience (par exemple dans la teneur en NaCl des solutions).

## CONCLUSION GÉNÉRALE

De l'étude à laquelle nous nous sommes livrés, on ne peut donc, ainsi que nous l'avions laissé prévoir, tirer que des conclusions qualitatives dont certaines sont apparues au cours de cet exposé.

Nous allons maintenant les résumer et les compléter.

— Les échantillons provenant d'un tissu donné contiennent un mélange de macromolécules de masses différentes dont la moyenne en poids, donnée par la diffusion de la lumière, dépend de la méthode d'extraction employée, sans qu'on sache encore exactement comment. Si l'on admet qu'aucune agrégation ne se soit produite pendant les opérations d'extraction, les solutions contiennent seulement des débris des macromolécules originelles et, dans ces conditions, la méthode optima de préparation est celle qui donne la masse la plus élevée.

De l'ensemble des données connues de l'auteur, c'est la méthode de Vendrely (16) qui donne, de ce point de vue, le meilleur résultat avec une masse molaire moyenne de 16.5 millions.

— Si l'on admet que l'ADN extrait se présente sous la forme d'une double spirale constituée par deux chaînes liées par des ponts d'hydrogène, comme le proposent Crick et Watson, un filament linéaire et rectiligne d'une telle masse aurait plus de 8 microns de longueur.

Pour qu'il puisse se loger dans les bandes chromosomiques, il est indispensable qu'il soit plié fortement sur lui-même en un certain nombre de points.

Le modèle de Crick et Watson présente, autant qu'on puisse s'en rendre compte, une rigidité trop grande pour qu'une semblable opération soit possible, à moins que, de loin en loin, se trouvent sur le filament des petits domaines de moindre résistance, donc de structure différente.

On retrouverait ainsi un modèle s'apparentant à celui qui a été présenté par Dekker et Schachmann (26), sans toutefois que celui-ci, de ce point de vue au moins, s'impose nécessairement.

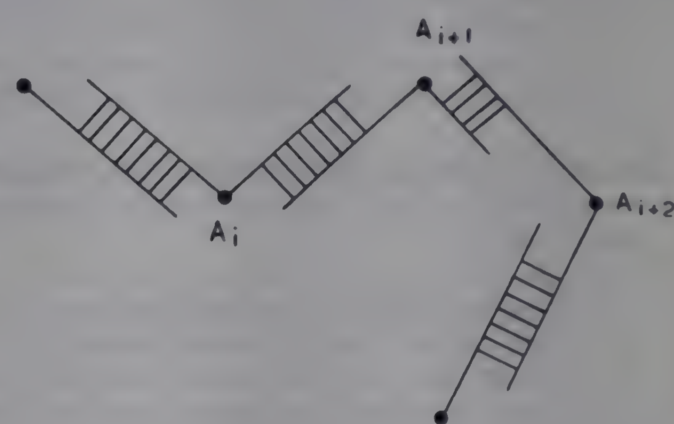


FIG. 10. — Élément de macromolécule coudée, selon Sekker et Schachmann (26).

En particulier rien ne prouve que les charnières $A_i$ à cause des empêchements stériques permettent un pliage suffisamment serré.

On pourrait tout aussi bien admettre que les zones « souples » soient constituées par des éléments de chaînes unifilaires d'une constitution chimique différente, par exemple des résidus protéiques indosables pratiquement, ou encore que, dans certaines régions de la double spirale il existe un certain désordre local dans la disposition relative des bases puriques ou pyrimidiques.

C'est pourquoi nous préférons sans aucunement rejeter la suggestion de Dekker et Schachmann, schématiser le modèle sous un aspect plus général où les zones hachurées (figure 10) correspondraient encore à des éléments ou groupes d'éléments en double spirale, mais où les portions unifilaires ne seraient pas constituées nécessairement par les segments :

$$-\mathrm{O}-\overset{\overset{\displaystyle \mathrm{O}}{\|}}{\underset{\underset{\displaystyle \mathrm{OH}}{|}}{\mathrm{P}}}-\mathrm{O}-$$

Cette manière de représenter les macromolécules d'ADN évite un inconvénient qui semble se présenter pour le modèle peut-être trop précis de Dekker et Schachmann.

Ces auteurs, considérant que les régions d'articulation, dans leur modèle sont des liaisons diphosphoesters, estiment que la dégradation thermique à 100° C. ne peut s'effectuer par rupture de ces liaisons d'énergie élevée. Ils admettent de préférence que la dégradation de la chaîne est produite par rupture de certains groupes de liaisons hydrogène entre bases. S'appuyant alors sur les travaux de Thomas (28) selon lesquels l'absorption ultraviolette pour $\lambda = 260$ mμ augmente quand ce phénomène se produit, ils admettent que la dégradation faite par ce mécanisme s'accompagne d'une augmentation correspondante dans l'absorption de la substance dans l'ultraviolet.

Or, ainsi que nous l'avons observé, nous avons pu voir pour la masse des échantillons S VIII et S V de 5 millions à 300 000 sans variation notable de l'absorption.

Sans que ce fait soit en contradiction absolue avec le modèle de Dekker et Schachmann, il suggère néanmoins des possibilités autres et, en particulier, une nature différente des régions d'articulation où, pensons-nous, se produiraient principalement les ruptures.

En résumé, c'est cette chaîne complexe correspondant néanmoins au schéma général de la figure 10 qui, si elle existe *in situ*, se trouverait repliée régulièrement sur elle-même en enfermant dans ses méandres les protides qui y sont liés.

Au cours de l'extraction, elle subirait des déformations autour des régions d'articulation, en même temps qu'une certaine dégradation.

Nous pensons qu'il ne convient pas dans cet exposé, et dans l'état actuel des faits expérimentaux, de nous livrer à des spéculations plus ou moins hasardeuses pour préciser ce modèle très général, mais nous considérons cependant que les résultats modestes obtenus jusqu'ici pourront prochainement, surtout s'ils sont précisés par des expériences rigoureuses, nous conduire à une rotation satisfaisante du difficile problème que nous nous sommes posé.

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## Addendum

(*Reçu le 28 juillet* 1955)

*Note sur l'action de la chaleur sur les solutions d'ADN*

*Effet de la concentration saline.* — Les solutions ont été portées à 100° C. pendant 15 minutes. Elles contenaient toutes $2 \times 10^{-4}$ g./ml. d'ADN, qui est la concentration minima permettant les mesures de diffusion de la lumière. Il faut remarquer que l'effet de la concentration en ADN devra être étudié systématiquement.

Seuls, jusqu'ici, ont été mis en expériences les échantillons S V et S VIII de masse commune égale à $6 \times 10^6$. Les résultats sont donnés dans le tableau II.

TABLEAU II

| Concentration en NaCl (M) | S V | | S VIII | |
|---|---|---|---|---|
| | $M(\times 10^{-6})$ | $r_g$ (Å) | $M(\times 10^{-6})$ | $r_g$ (Å) |
| — | 0.28 | 275 | 0.35 | 260 |
| 0.2 | 2.5 | 850 | 1.5 | 700 |
| 1.0 | 3.6 | 1130 | 7 ± 1 | 1230 |

Durée : 15 minutes. Température : 100° C.

On constate pour les deux échantillons, l'existence d'un effet protecteur du chlorure de sodium. Néanmoins, l'effet du chauffage a été de dégrader l'ADN, sauf dans le cas de la solution de S VIII en présence de NaCl M.

*Effet de la durée.* — Les mesures ont porté uniquement sur la solution de S VIII en NaCl M.

On a chauffé pendant 30 minutes, puis pendant 90 minutes. La masse et le rayon de giration sont restés constants dans les deux cas, et cela dans les limites de précision de la mesure ($\pm 10$ % sur les valeurs données).

On a, au cours de ces expériences, étudié le spectre d'absorption ultraviolette des solutions. On a constaté que le maximum d'absorption restait compris entre les longueurs d'onde de 2580 et 2600 Å. On n'a trace d'aucune bande d'absorption à 2800 Å, ce qui prouve que les bases n'ont pas été libérées.

Dans tous les cas, on constate une augmentation du coefficient d'absorption à 2600 Å, sous l'influence du chauffage, même, nous y insistons, lorsque la masse ne change pas, comme on l'a signalé pour S VIII en solution NaCl M. Dans ce cas, l'augmentation est de l'ordre de 8 % après 30 minutes de chauffe.

L'étude détaillée du phénomène présente des particularités qu'il serait prématuré de signaler dans l'état actuel des travaux. Ainsi nous contentons-nous de souligner ici que l'augmentation d'absorption dans la bande des 2600 Å se fait même s'il n'y a pas changement de masse selon la concentration de la solution en NaCl.

On comprend alors que, selon la concentration en NaCl employée, deux expériences faites avec une même durée de chauffe et à la même température, puissent ou non conduire à une diminution de la masse moléculaire de l'ADN.

### *Note sur la nature des régions souples*

Il est important de signaler ici une suggestion faite par Ambrose (Ambrose, communication privée), et selon laquelle la double spirale est détordue en certaines régions (figure 11) où, naturellement, elle est alors plus souple.

FIG. 11. — Zone de pliage (selon Ambrose).

Dans ces conditions, la molécule d'ADN comporterait une suite de segments rectilignes correspondant chacun au modèle de Crick et Watson, séparés par des zones « en échelle » lui permettant de se plier.

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# The characterization, denaturation and degradation of desoxyribose nucleic acid

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(*Received* 9 *September* 1955)

The past two years have seen a substantial expansion in the investigation of desoxyribose nucleic acid (DNA). Much of this effort has been catalyzed by the Watson-Crick model (1) for the short range configuration of the DNA molecule and it has already become clear that numerous experimental findings are explicable in terms of this concept. In addition there has been a widening use of properly prepared samples and a growing recognition of the chemical diversity of molecules within a given sample as shown by recent successes in fractionation (2, 3, 4).

In this report attention is focused on the physical chemical studies of DNA in dilute solution with a myopic concern with work done in this laboratory. Studies of this type have generally been rewarding if two key conditions were met : first, the samples employed must have been carefully prepared and secondly, the concentration range must have extended well below 0.01 %. The requirements of a careful preparation seem to have been met by Signer and Schwander (5), Kay, Simmons and Dounce (6) and particularly by recent improvements due to Simmons (7) in which a 30 % solution of sodium xylene sulfonate is used to dissociate the nucleoprotein and inactivate any enzyme. In all cases the storage of the glands (or other source tissue or culture) in dry ice immediately upon slaughter is essential. The condition of using high dilutions for investigation is made necessary by the extremely large volume of solution occupied by a DNA molecule. Indeed upon rotation about its center of gravity it appears that the DNA molecule cuts out a volume of about ten thousand times its own molecular volume on a dry basis. Hence the requirement of working below 0.01 % because it is only here that the individual molecules can move independently and forced interpenetration no longer exists. This is the condition that is met at about 1 % for typical asymetric proteins like fibrinogen, and at about 0.1 % for typical synthetic polymers of molecular weight of 1 million or more. Investigations in which these two essential conditions have been met have only taken place within the last five or six years and in almost every instance take precedence over earlier work.

### *Characterization*

Due chiefly to the foregoing limitation on the concentration at which measurements can be made, the only macromolecular techniques which have yielded reliable information on DNA are light scattering, intrinsic viscosity and sedimentation rate. From light scattering one obtains the weight average molecular weight and information concerning the size and shape. Without

ambiguity we obtain the radius of gyration of the molecule but with the additional knowledge that it is a highly extended chain we can interpret this in terms of the mean end-to-end distance since in such a case this distance is $\sqrt{6}$ times the radius of gyration. The intrinsic viscosity must be determined by first measuring the relative viscosity at a series of rather low gradients and then extrapolating this type of result at several concentrations to zero gradient. From these values at zero gradient the intrinsic viscosity is determined in the usual way. Provided the shape of the DNA molecule is that of a highly extended chain the intrinsic viscosity, $[\eta]$, can be interpreted either in terms of the mean end-to-end length, $\overline{R^2}$, and the molecular weight, $M$, or in terms of the frictional constant and $\overline{R^2}$. Thus the intrinsic viscosity gives us information essentially about the size of the DNA molecule in solution. Indeed, as an approximation, the intrinsic viscosity in units of ml./g. tells us the effective volume of DNA in solution. By way of contrast the sedimentation constant, $S_0$, is, for a highly extended chain or a rod, primarily sensitive to the cross-sectional diameter of the chain or rod. Its value reflects this cross-sectional dimension but it can be alternatively interpreted in terms of the frictional constant. As a consequence it can be combined with the intrinsic viscosity to provide, for various model particles, relations in which the frictional constant has been eliminated and in which only directly measurable constants remain. One of the simplest models that represents a reasonable model for DNA is the free-draining coil. In this case

$$\frac{S_0\,[\eta]^{1/3}}{\overline{R^2}} = \frac{(1 - \overline{V}\rho)}{3600\;\eta_0} \qquad (1)$$

where $\overline{V}$ is the partial specific volume, $\rho$ is the density and $\eta_0$ the viscosity of the solvent.

The number of reported light scattering determinations on DNA samples is now of the order of fifty. The earliest values (8, 9, 10) of the molecular weight of calf thymus DNA were in the range of 3.5 to 7.7 million and with the improvements in preparative procedures this has been narrowed to 5.8 to 7.7 million (11). The radii of gyration average about 2200 Å and are in this narrow range proportional to the square root of the molecular weight. In terms of $(\overline{R^2})^{1/2}$ we have 5500 Å and this serves as a rough measure of the diameter of the volume occupied by one DNA molecule.

The intrinsic viscosity (at zero gradient) for these samples ranged from 31 to 72 (in units of 100 ml./g.) for the range of 3.5 to 7.7 million molecular weight. These figures reflect the proportionality of intrinsic viscosity to molecular weight. This is the behaviour expected for samples having the configuration of a highly extended, free-draining chain and differing from one to another only in the average chain length.

The sedimentation constant is best evaluated by the extrapolation to zero concentration of the reciprocal of the measured value. By carrying measurements to lower and lower concentrations the published values of $S_0$ have risen from 13 to 22.5 (7, 12) over a five year period and the recent values may be accepted with confidence (*). These more recent values for $S_0$ do not show a variation among samples of different molecular weight indicating thereby that the molecular cross-section in the different samples remains constant. The cross-sectional diameter to be associated with this value of $S_0$ is 25 Å.

From these observations it is clear that the DNA molecule in solution is a highly extended chain. Its molecular weight lies in the range of 7 million and the variations in molecular weight of different samples correlates closely with the size as determined from light scattering or viscosity and this indicates the same local chain stiffness in the different samples. At the time of the first light scattering measurements the point was made that a single polynucleotide chain of the observed molecular weight would be much more tightly coiled than that indicated by the observed size and that there may therefore be several strands making up the molecule. The equatorial reflections of the early X-ray diagrams pointed in the same direction and we have now the further support of the sedimentation result and direct electron microscopy (13). All of these observations are consistent with a diameter of the size required for the Watson-Crick model and as a result there seems little doubt that this represents the detailed local structure in solution as well as it does in the crystalline state.

The question naturally arises concerning the extent to which DNA samples from other sources resemble the data obtained on calf thymus preparations. We have had the opportunity of examining several samples prepared from different sources by Dr. N. S. Simmons and although these results are quite inadequate for forming a broad generalization they do perhaps indicate the direction to which the answer to such a query will be found to lie. Measurements on DNA from pneumococcus showed (14) it to have a molecular weight of 7.7 million and intrinsic viscosity of 72. Measurements on DNA from chicken erythrocytes yielded (15) 4.2 million for the molecular weight and 38 for the intrinsic viscosity. Herring sperm DNA obtained from Dr. P. Alexander was found to have a molecular weight of 5.7 million (14). Unpublished studies (16) of several years ago on DNA from T2 bacteriophage led to molecular weight values of about 10 million but the result was in doubt because of the residual protein that could not be removed. Sedimentation studies (17) show it to have the same sedimentation characteristics as calf thymus DNA but $S_0$ itself was not determined. Thus one finds some basis for the conjecture that the DNA over the whole biological scale has similar macromolecular properties.

### *Denaturation*

If by denaturation one means the breakdown of a unique and specific structure then the partial or complete breaking apart of the two strands making up the DNA molecule according to the Watson-Crick structure should receive this designation. As in the case or proteins the breakdown of the specific repeating structure of DNA

(*) The value of $S_0$ given in (12) is 21. The measurements of N. S. Simmons were carried out at still lower concentrations and yielded an extrapolated value of 22.5.

should be characterized by a large entropy of activation. In addition to thermally induced denaturation, proteins are readily denatured by acids, bases and certain agents such as urea. It is of interest to see if nucleic acid responds in a similar manner.

In contrast to most globular proteins the denaturation of DNA should be accompanied by a striking fall in the viscosity of its solution. In retrospect it can now be seen that the first observation of the denaturation of DNA by acid and base was observed by Creeth, Gulland and Jordan in 1947 (18). They found the viscosity to remain constant from pH 5 to 11 but to drop precipitiously beyond these limits. At that time it was thought that this was evidence for a degradation or the dissociation of a micellar structure but in a detailed study of this situation (19) on the acid side it was found that, provided the pH adjustments are made in a way that avoids excessive local concentration of strong acid, no change in molecular weight occurred but that instead there was a substantial contraction in the molecule. This is viewed as the result of the two chains taking up their more probable configurations when relieved from the many restraints imposed by the hydrogen bonding of the native structure. The observation that the molecular weight does not fall is indicative of the two strands being held together either by residual hydrogen bonds, by molecular entanglements or by occasional bonds, such as metal-ion chelation, that has not yet been identified. With the careful adjustment of pH it was found that the fall in viscosity did not occur until a pH of 3.1 (20). The original explanation of Creeth, Gulland and Jordan in terms of the sudden breakup of hydrogen bonds by the acceptance of protons by the groups involved remains valid.

The behavior of DNA in the presence of base has been recently studied (21) and found to be very similar to that found for the acid. The fall in viscosity occurred in the vicinity of pH 11.8 and no change was observed in the molecular weight at pH 12.2 over a period of days. The base denaturation, as well as the acid denaturation, is irreversible if the denaturation is allowed to reach completion. In both cases the product has an intrinsic viscosity of 10 to 12 fold lower than the native material and the radius of gyration is reduced to 900-1000 Å. At pH 12.2 it is expected that all of the hydrogen bonding groups are titrated and consequently the failure of the strands to separate here is indicative of their being held together for other reasons, perhaps that of entanglement.

Upon heating neutral, saline solutions of DNA no change in molecular size occurs until a critical temperature range is reached (22, 23). In the older samples (11) this was at 75 to 80° C.; in the new preparations it was ten degrees higher. The energy of activation in the later case was also considerably higher — 93 kcal. in contrast to 36 kcal. for the older samples. The heat denatured product again showed no change in molecular weight and the molecular size had decreased to that found by acid and base denaturation. The presence of 8 M urea and other protein denaturating agents lowered the denaturation temperature as much as 17° C. but did not alter the entropy of activation for the process, this remaining at about 220 e.u. A model which accounts for this denaturation process has been worked out (24) and requires that the transition state correspond to the opening up of a gap in the Watson-Crick structure corresponding to the breaking of about 15 successive hydrogen bond pairs. The denatured material is then viewed as the result of inducing many gaps of this size in the native structure and the contraction is thought to be due to the increased freedom of rotation produced at each gap in the structure Smaller gaps are believed to form but because of their small size they are not stable and heal to re-form the original local hydrogen-bonded structure.

In all of these denaturating reactions the optical density measured at pH 7 in 0.2 M NaCl and at room temperature is found to rise by the same amount, 14 %. It has been recognized for some time, especially by Hotchkiss, that this characteristic rise upon denaturation was a reliable test to determine if a given sample is in the native configuration. We have found the temperature dependence of the intrinsic viscosity as determined by the change observed in one hour heating periods at various temperatures up to 100° C. to be an even more sensitive index. This is indeed a refinement of the procedure employed by Zamenhoff (24, 25) in which he was able to show a good correlation between the loss of transforming activity of DNA and its extent of denaturation. In addition a characteristic change in the infrared spectra of DNA when denatured has been observed by Blout and Lenormant (26). As further evidence of the change in local configuration that accompanies denaturation a change, of 220° in the optical rotation may be sited (24).

Against the background sketched above it is of interest to examine two provocative papers that have appeared within the last year. In one of these Dekker and Schachman (27) claim that heating 0.005 % of DNA at 100° C. for 15 minutes causes a reduction in the sedimentation constant from 20 to 6. This together with the drop in viscosity already discussed leads them to conclude that the molecular weight has dropped a hundred-fold. This is, of course, in contradiction to the view developed above in which the molecular weight would not be expected to change. The implications of this point are important for it is on this evidence that the authors propose that native DNA is a two-stranded structure but that there are many non-coincident breaks in each of the strands.

The sedimentation constant of heated material had been determined in this laboratory (24) and had been found to be about 30 and this increase rather than a decrease in $S_0$ on DNA prepared by Simmons' procedure has been confirmed independently (28). Upon taking up the discrepancy with Professor Schachman it was found that their published results referred to heat treatment of salt-free solutions. We have confirmed that indeed the effect is different there and suspect that primary valence bonds are being broken due to the additional weakening provided by the electrostatic repulsions of the phosphate groups. Since investigation of this situation does not appear to provide information on the native DNA structure we have sought to interpret the results obtained in the presence of salt (23) and find that the simultaneous changes of the sedimentation constant and intrinsic viscosity brought about by heating saline solutions of DNA do not require any assumption

of molecular weight changes. For example, using the free draining coil model the collection of constants on the left of equation one have values of 0.030 and 0.021 for native and heat denatured DNA respectively.

The other paper of interest is that of Alexander and Stacey (29) in which they claim that light scattering measurements show a halving of the molecular weight of herring sperm DNA upon being subjected to any of three different treatments : (1) exposure to pH 2.2 for 3 minutes, (2) exposure to 4 M urea and (3) heating to 100° C. for 15 minutes. Whereas the evidence for the two stranded structure of DNA we now consider overwhelming and as a consequence the two strands should be separable at least in principle, it is our belief that none of the foregoing treatments produce this result. The objection to the acid treatment lies in our own investigation of acid degradation (20) (discussed in the next section) whereby it was clear that rapid hydrolysis and in particular very rapid loss of purines would account for the observed decrease in molecular weight. We find it possible to prepare isolated polynucleotide chains by acid treatment but they have suffered some phosphateester bond scission and have lost some purines. We have had the opportunity to examine the other two methods on herring sperm DNA kindly furnished by Dr. Alexander. The urea treatment at 4 M does not in our experiments show any change in molecular weight when measured after the removal of urea. Moreover the radius of gyration is still the same and the characteristic increase in optical density at 260 mμ occurs upon heating. Both of these observations show that the Watson-Crick structure is intact after the treatment with 4 M urea and support our original finding that urea even at 8 M has no effect on DNA at room temperature. It appears that Alexander and Stacey's observations as well as those of Conway and Butler (30) were made on material that had become at least partially denatured or degraded prior to the experiment. With regard to the third method, that of the 100° C. heat treatment, we must report that here too we find no change in molecular weight on the herring sperm DNA in keeping with our observations on thymus DNA. Consequently we view with some misgivings the claims that intact polynucleotide strands can be prepared by such mild procedures.

### *Degradation*

By degradation we refer primarily to scission of the polynucleotide chains and in this sense two methods of hydrolytic cleavage of the phosphate ester bond have been examined in this laboratory and found to be interpretable in terms of the double chain structure for DNA.

Light scattering offers a particularly useful way of studying the degradation of DNA because it provides the weight average molecular weight as a continuous function of time. The way in which the weight average molecular weight, $M$, varies with the random scission of a chain molecule has been long known. If the original molecular weight distribution is the most probable one (sometimes called the random distribution) the reciprocal of $M$ increases linearly with time. Other initial distributions give rise to small degrees of curvature during the early part of the degradation. The molecular weight decay of a double chain due to random scission of single chain bonds will display quite a different behavior because a fall in molecular weight will only occur when both chains have been cut opposite each other or within a given number of chain units of each other. The solution of this problem (20) shows that the plot of $1/M$ against time will have an initial slope of zero and curve upward in a fashion characterized by the number of secondary bonds that will hold cut chains together.

The first hydrolytic degradation investigated (20) was that induced by acid. The rate of degradation became measurable at about 2.7 and increased steadily with decreasing pH. We chose pH 2.6 where the $M$ was decreased to half in about two days. Under these conditions it was found that, following the immediate contraction of the DNA molecule in the acid media, three types of degradation may be delineated. The phosphoester bonds break at the rate of about one bond per DNA molecule per 10 hours. Secondly, purines are liberated at about 200 times this rate. Finally, there is substantial evidence that during the early part of the degradation the two original polynucleotide strands making up the native molecule are held together at a few places by what are thought to be residual hydrogen bonds or intact sequences of hydrogen bonds. The kinetic data interpreted within the framework outlined in the previous paragraph indicate these regions are separated initially by about 300 nucleotide units. However, these also decay with time and disappear after about fifty hours. Thereafter the process is simply that of the random scission of single polynucleotide chains which are continuing to lose purine groups.

In contrast to the acid degradation which proceeds after denaturation it was of interest to examine the enzymatic degradation which takes place under normal conditions. Previous work on this reaction had been done at such high enzyme concentration that little was known concerning the early stages of the reaction. The light scattering technique was most suitable in this region and a number of preliminary observations were carried out in 1953 by Reichmann (31). His observations established that (*a*) there is initially a retardation in the molecular weight decay and (*b*) while the molecular weight falls the configurational properties of the fragments seem to be the same as the original material. When the statistics of degradation of a double chain were available it was evident that the molecular weight decay data fitted the model of a double chain degrading by random single chain scission.

The demonstration that the mechanism of the degradation was of the type indicated required an independent determination of the number of single chain bonds broken as a function of time. This has just been done by Thomas (32) using a pH-stat which maintains the pH in a light scattering cell at a constant value by the regulated supply of ammonia carried in a stream of nitrogen. Such an arrangement allows the simultaneous measurements of the weight average molecular weight by light scattering and the number of phosphoester bonds scissions by the consumption of ammonia. The analysis of the results shows that indeed the molecular weight decay is quantitatively accounted for by the random scission of single chains proceeding at the

observed rate and that two pairs of hydrogen bonds are required to keep cut fragments unitied. That is, the molecular weight will fall if cuts in two chains are coincident or displaced from each other by one or two chain units (nucleotides). The changes in viscosity at 25° C. are found to parallel those of molecular weight : this indicates that the interchain hydrogen bonds are sufficient to support the structure even when occasional internucleotide bonds are broken. Thus, for example, both the molecular weight and the viscosity show only a 20 % decrease from their original values when each molecule has received an average of 100 cuts. One result of this work is that it makes possible the introduction of known amounts of phosphoester bond scission in transforming principle with a view to finding out if this is sufficient for inactivation.

It is a pleasure to acknowledge our indebtedness to Dr. N. S. Simmons for many of the DNA samples used in the work reported above and for much counsel and advice. Most of the work originating in this laboratory was supported by a research grant (C-2170) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

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## The sedimentation behaviour of DNA and the effect of heat and X-rays

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(*Received* 31 *July* 1955)

We have made many observations of the sedimentation constants of DNA at high dilutions using an optical absorption method for observing the boundary. The details of the method and a discussion of the validity of the distribution curves will be described elsewhere (1). I need only point out here that a very wide distribution of sedimentation constants is observed with all specimens of DNA examined, although the spread of the sedimentation constants is greater in some cases than others. Some typical examples are shown in figures 1 and 2. This is evidence of sedimentation heterogeneity, although its significance at present is not clearly known. It has been shown however that the sedimentation constant of long cylinders of constant diameter does not depend to any great extent on their length (2), so that the results cannot be accounted for as due to varying length in a system of cylindrical particles of constant diameter. No unambiguous conclusions can be drawn from other models, but at least it can be said that with a random coil with or without draining $M$ & $S^3$ and the variation of molecular weight for sedimentation constants varying from 10 to 40 would have to be considerable. Particles of varying effective diameter would be produced by (*a*) aggregation of different numbers of elementary

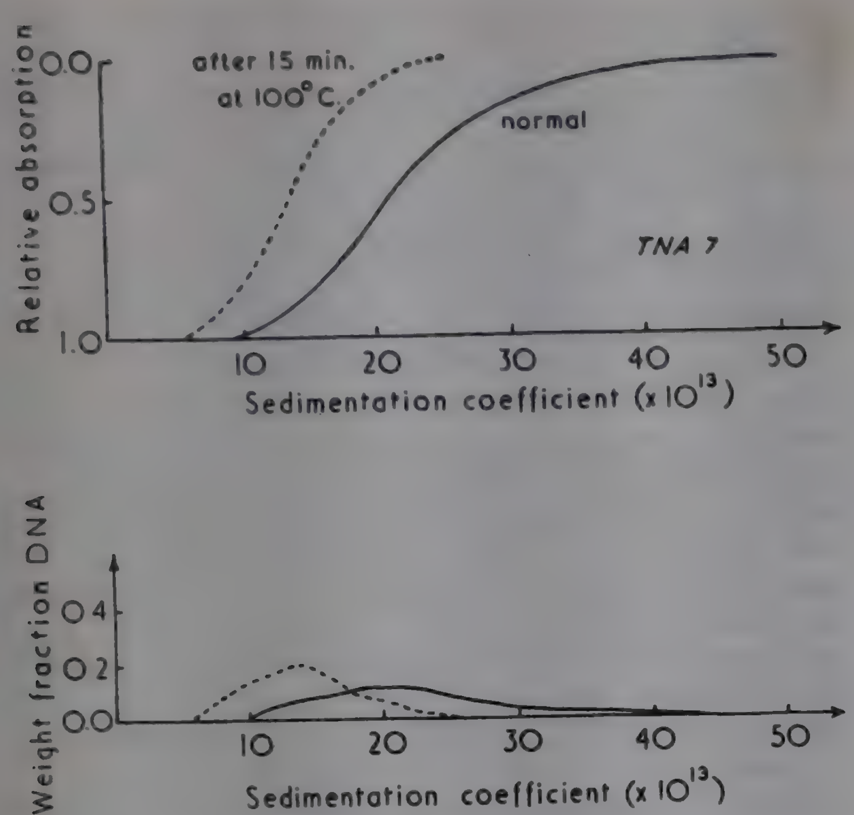


FIG. 1. — The effect of heating calf thymus DNA ($5 \times 10^{-5}$ g./ml. in 0.2 M NaCl).

particles ; (*b*) the fraying out of a twin thread to different degrees (see figure 3).

In an attempt to distinguish the first we studied the result of dispersing the DNA in 0.01 N NaCl instead of in water, before bringing it to the final salt concentration. The general result of this work was that the final sedimentation curve was independent of the method of dispersion, and this has been confirmed by viscosity measurements, made after different times of dispersion in water. Therefore if there is aggregation it does not depend within these limits on the mode of preparation of the solutions.

The effect of heat on solutions of DNA has also been studied. Whereas Schachman and Dekkar (2) found a marked decrease of $S$ on heating and Doty and Rice (3) an increase, our work on several specimens (including Doty's) shows either no great change of $S$ or a decrease of about 20 % (see table I). It might be noted that in cases where there is no change in the mean value of $S$, the distribution of sedimentation constants is affected by heat in that there is an increase of both low values and high values of $S$. In every case the viscosity is greatly reduced by heat (table I) and the only simple explanation of a decrease of sedimentation constant is that there is a decrease of molecular weight. It was found that the samples of DNA prepared by the detergent method of Dounce, Simmons and Kay (4) are less changed on heating than those made by the enzyme method (5)

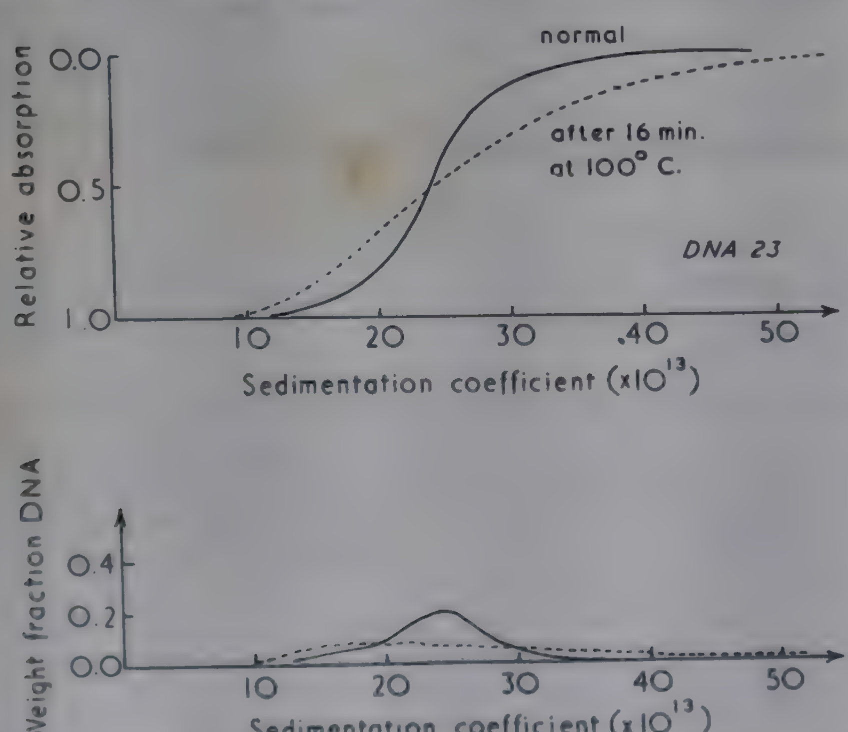


FIG. 2. — The effect of heating calf thymus DNA ($5 \times 10^{-5}$ g./ml. in 0.2 M NaCl).

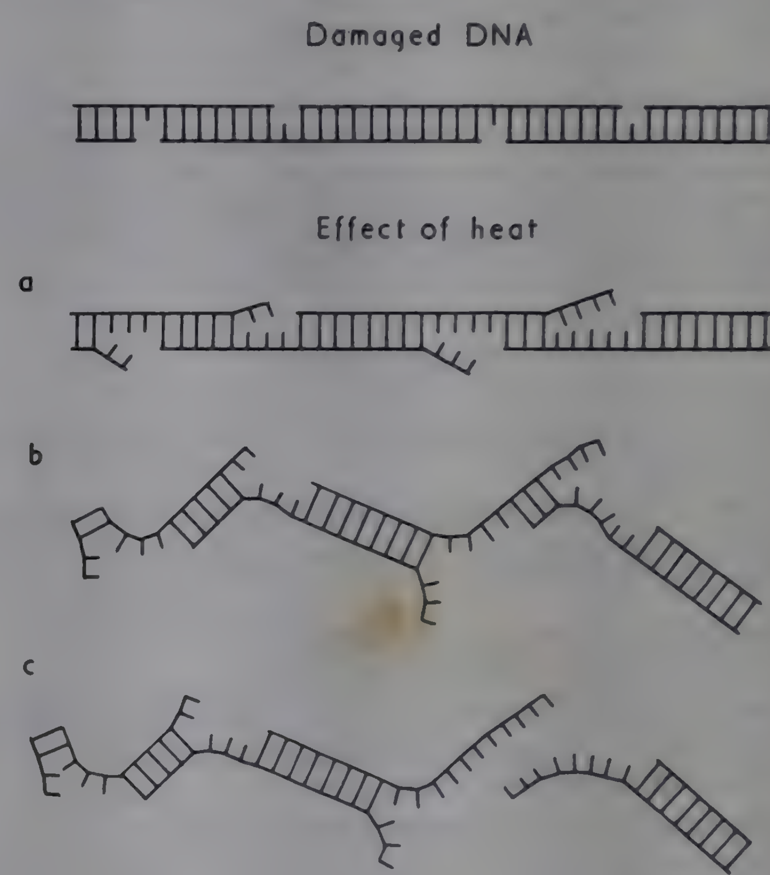


FIG. 3.

TABLE I

*Effect of heating (100° C. for 15 min.) on dilute DNA solutions*

| Preparation | Concentration (%) | Weight average sedimentation coefficient ($S \times 10^{13}$) | | Specific reduced viscosity in 0.2 N NaCl (ml. $\times$ g.$^{-1}$ $\times$ $10^{2}$) | |
|---|---|---|---|---|---|
| | | Before heating (in salt) | After heating | Before heating | After heating |
| TNA 7 | 0.01 | 20 | 14.4 | — | — |
| | 0.005 | 23.4 | 15.8 | 4.5 | — |
| TNA 15 | 0.01 | 19.6 | 15.2 | 5.2 | 0.3 |
| TNA 23 | 0.01 | 20.0 | | | |
| | 0.005 | 24.0 | 24.0 | 1.4 | 0.1 |
| TNA 23 (*) | | | | 27.0 | 0.7 |
| DNA-SB-11 | 0.01 | 20.8 | 20.0 | 7.2 | 0.43 |

(*) Heated in water only, not in 0.2 N NaCl.

which show appreciable degradation. It is likely that in the latter some nucleotide bonds have been broken by the action of traces of deoxyribonuclease, present in the chymotrypsin.

In order to confirm this possibility the specimen of DNA 23 which was little changed by heating was treated with X-rays in aqueous solution (9000 R), since it is known that this treatment is capable of breaking the nucleotide chains (6). After this treatment it was found to be more susceptible to the action of heat (table II).

TABLE II

*Effect of X-rays on heat stability of DNA preparation (DNA 23)*

| | Mean sedimentation constant | | Mean sedimentation constant after 15 min. at 100° C. | |
|---|---|---|---|---|
| | 0.01 % | 0.005 % | 0.01 % | 0.005 % |
| Original . . . . . . . | 20 | 24 | — | 24 |
| 4 h. after X-rays (9000 R) | 16.8 | 20 | | |
| 24-26 h. after X-rays (9000 R) . . . . . . | 13.8 | 20 | | |
| 14 days after X-rays (9000 R) . . . . . . | 16 | 19 | 10 | 12 |

It was thought possible that traces of detergent remaining in the preparation may have a stabilizing effect. This has been tested by adding the detergent to a heat sensitive preparation (at a concentration equal to that of the DNA). It had no effect on the sedimentation curves before and after heating.

It is also possible that aggregates may occur in the DNA preparations owing to the presence of small amounts of protein. This might also affect the behaviour on heating as additional cross-linking could be caused by the reaction of the protein with DNA particles. In order to test this histone was added to DNA to the extent of 1 % of the DNA present. This had no effect either on the original sedimentation curve or on that observed after heating.

Since a great decrease of viscosity occurs even when the change of sedimentation constant is small, it is difficult to avoid the conclusion that a collapse of the particle leading to a smaller dimension occurs as in figure 3*b*. This collapse will originate from flaws caused by breaks in the nucleotide chains, which are present to different extents in the different preparations. But it can be seen that if two breaks in the twin fibres are sufficiently close together the breakage of hydrogen bonds in this region will cause a rupture of the particle as in figure 3*c*. So we have two effects which influence the sedimentation constant in opposite directions (*a*) a folding up of particles leading to higher values of *S*, and (*b*) a breakage of particles leading to lower values.

These two effects may leave the average value unchanged. It is also possible that aggregation may occur by hydrogen bonding between 'loose ends' of different particles which happen to be near each other. This would clearly occur with a greater frequency the greater the concentration and the lack of complete agreement of different experiments may well be due to the heating experiments having been performed at different concentrations.

It should also be noted that in some data given by other authors uncertainties may be present owing to extrapolation of sedimentation constants and viscosities from too high a range of concentrations.

A number of rather contradictory observations have been made on the effect of urea on DNA solution. Earlier workers (7) found a marked effect of urea (4 M and above) on the viscosity both when present and after removal by dialysis. This was confirmed by Conway by measurements at low concentrations in the Couette viscometer (8). Pain has repeated this work and still finds a decrease in viscosity at low concentrations and small shear rates, and Shooter found that after removal of the urea by dialysis the sedimentation coefficients fall to lower values (unpublished observations). On the other hand Doty and Rice (3) suggested that urea (8 M) has no effect on the viscosity at ordinary temperatures, but causes a lowering of the denaturation temperature.

This discordance probably arises from the different shear rates at which observations were made. In the observations of Conway and Pain (shear rate 0-5/sec.) marked interaction effects occur at all except the lowest concentrations and urea appears to eliminate these. At a shear rate of 30/sec., this particle interaction is hardly apparent and the effect of urea is small. It thus appears that the effect of urea is in the first instance on particle interaction and is similar to the effect of salts. The reason for this is not clear; but it must be observed that after the removal of the urea by dialysis permanent modification of the DNA is observable.

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# Propriétés hydrodynamiques des solutions diluées d'acide désoxyribonucléique

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(*Reçu le* 9 *septembre* 1955)

L'étude des solutions très diluées d'acide désoxyribonucléique (ADN) par ultra-centrifugation révèle l'hétérogénéité des échantillons du point de vue des constantes de sédimentation. Lorsqu'on effectue des mesures à des concentrations de l'ordre de 0.02 à 0.005 g./100 ml., on observe un applatissement considérable de la figure de sédimentation en fonction du temps. En première approximation, on peut estimer que pour une constante de sédimentation moyenne $S$ de $30 \times 10^{-13}$ c.g.s., la dispersion est de 15 à $45 \times 10^{-13}$ c.g.s.

Shooter et Butler (1) ont montré récemment qu'ils observaient également une polydispersité importante de leurs échantillons. Si on suppose que la configuration des molécules d'ADN est approximativement constante en fonction des masses moléculaires $M$, il faut admettre une polydispersité très importante. La relation entre $M$ et $S$ n'ayant pas été établie à partir de fractions plus ou moins homogènes d'ADN, on ne peut estimer que l'ordre de grandeur de la dispersion des masses en admettant que l'exposant de la relation : $S = K.M^{\alpha}$ est du même ordre ($\alpha = 0.33$) que pour les dérivés cellulosiques qui ont un comportement analogue à l'ADN tant du point de vue hydrodynamique que du point de vue de la diffusion moléculaire de la lumière. Sur cette base, on trouverait une polydispersité des masses variant de $0.7 \times 10^{6}$ à $20 \times 10^{6}$ en admettant que le poids moléculaire est égal à $6 \times 10^{6}$ pour $S = 30 \times 10^{-13}$ c.g.s. Une telle polydispersité aurait une répercussion importante sur toutes les mesures dépendant de la masse. Il se pourrait évidemment que les molécules d'ADN aient, à masse égale, des configurations variables dues à des pourcentages différents de « dénaturation » (Doty et Rice, 2).

Les mesures de biréfringence d'écoulement indiquent également la probabilité d'une importante polydispersité ; on trouve en effet que les valeurs expérimentales des constantes de diffusion rotatoire $\theta$ augmentent appréciablement et d'une manière continue en fonction du gradient de vitesse $G$ ; le système serait donc polydispersé et constitué de particules relativement indéformables. On trouve, par exemple, que $\theta$ varie de 5 à 900 pour $G$ variant de 0 à 2000/sec. ce qui correspondrait à une dispersion de la longueur de 12 000 à 2000 Å et à une dispersion des masses moléculaires de $8 \times 10^{5}$ à $2.5 \times 10^{6}$ en admettant une structure de bâtonnets rigides pour ces molécules et une masse de 200 par unité de longueur. La dispersion serait encore plus grande si nous supposons, comme pour une pelote gaussienne, que la longueur calculée à partir de $\theta$ est approximativement proportionnelle à $\sqrt{M}$. Cette dispersion serait alors du même ordre de grandeur que celle estimée par ultra-centrifugation.

Le problème de la polydispersité de l'ADN ainsi que celui de la relation entre les dimensions et les masses moléculaires ne pourront être résolus avec certitude que si on parvient à préparer des fractions relativement homogènes d'ADN ; ce problème est identique à celui qui se pose en chimie des polymères de synthèse.

En relation directe avec ce qui précède, on peut se demander si la polydispersité de l'ADN est due uniquement à une coupure de chaînes au cours de l'extraction des tissus ou si elle n'est pas due à une certaine agrégation provenant d'une déprotéinisation insuffisante. Il est possible qu'un pourcentage minime d'histone (environ 1 %) soit suffisant pour provoquer une agrégation des molécules d'ADN en particules très grandes et de configurations très différentes.

Il est peut-être intéressant de mentionner quelques résultats que nous avons obtenus sur un ADN préparé à partir de thymus de veau par la méthode de Kay, Simmons et Dounce (3) : déprotéinisation avec le dodécylsulfate de soude.

Les viscosités de cet échantillon mesurées en fonction du gradient de vitesse $G$ dans un viscosimètre capillaire spécial peuvent être extrapolées à gradient nul et à concentration nulle grâce aux relations suivantes :

$$\frac{1}{\eta_{\text{rel}}} = A + B\tau \quad \text{avec } A = \left(\frac{1}{\eta_{\text{rel}}}\right)_{\tau=G=0} \qquad (1)$$

$\tau$ est la tension de cisaillement maximum et a pour valeur « pression $\times$ rayon du capillaire : 2 fois la longueur du capillaire » ; les mesures sont faites avec $\tau$ variant de 0.3 à 3 dynes/cm² ce qui correspond à des $G$ de 10 à 100/sec.

En fonction de la concentration, les résultats extrapolés à $\tau = 0$, satisfont la relation suivante :

$$\frac{\log (\eta_{\text{rel}})_{\tau=0}}{c} = [\eta] \quad \text{pour } c \text{ variant de 0.004 à 0.05 g./100 ml.} \qquad (2)$$

Nous trouvons pour l'échantillon utilisé : $[\eta] =$ 6200 ml./g. dans NaCl 0.2 M comme solvant (pH = 6.5).

Les constantes de sédimentation $S$ mesurées dans le même solvant peuvent être extrapolées à concentration nulle de la manière classique en portant $1/S$ en fonction de la concentration $c$ (0.1 à 0.005 g./100 ml.). On trouve pour l'échantillon utilisé :

$$S = 29.4 \times 10^{-13} \text{ c.g.s.}$$

En combinant $S$ et $[\eta]$ on peut calculer le poids moléculaire suivant deux modèles extrêmes :

— le cylindre très allongé utilisant les formules de Perrin ;

— la chaîne statistique utilisant la relation de Mandelkern et Flory (4).

Nous trouvons ainsi une masse de $5.75 \times 10^{6}$ pour le cylindre très asymétrique non hydraté et de $13 \times 10^{6}$

pour la pelote gaussienne très hydratée. Remarquons que si on admet une hydratation variable du cylindre très asymétrique, mesurée par le rapport : volume moléculaire hydrodynamique/masse moléculaire réelle, on trouve des valeurs de $M$ qui augmentent de 5.75 à $7 \times 10^6$ quand on fait varier l'hydratation de 0.55 à 10.

La nucléoprotéine à partir de laquelle a été obtenu l'ADN a également été étudiée par viscosité et sédimentation. Si on calcule la concentration en nucléoprotéine sur la base de 3.8 % de phosphore, on obtient : $[\eta] = 8000$ ml./g. et $S = 27 \times 10^{-13}$ c.g.s. ; on peut donc à nouveau calculer les poids moléculaires pour les deux modèles en utilisant 0.65 comme volume spécifique partiel. On obtient ainsi une masse de $8.5 \times 10^6$ pour le cylindre très asymétrique non hydraté et de $19.2 \times 10^6$ pour la pelote gaussienne fortement hydratée.

Comme dans le cas de l'ADN on trouve une dispersion importante des constantes de sédimentation aux faibles concentrations.

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# The chemistry of the ribonucleic acids

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(*Received* 2 *May* 1955)

## *Introductory*

The ribonucleic acids have been known since 1889, when Altmann (1) first isolated yeast nucleic acid, which has probably been the most extensively investigated compound of this type. From this time until the appearance of the classical monograph of Levene and Bass (2) in 1931 'plant nucleic acid', as it became known, was investigated by a number of eminent organic chemists who established the general nature of the constituent parts, but who had no idea of the complexity of the material which they were investigating and little regard for its evident lability. The net result of these investigations was an oversimplified idea of the structure of ribonucleic acids, but fortunately one in which two very essential points were lacking. These served to keep a number of workers interested in the problem in spite of the fact that the main details appeared to have been well established.

The situation as Levene and his contemporaries saw it, and as it is set out in most textbooks even now, is as follows : ribonucleic acid is a compound which gives rise on hydrolysis to four major components, the nucleotides adenylic acid, guanylic acid, cytidylic acid and uridylic acid. Each of these nucleotides consists of a purine or a pyrimidine linked by an N-glycosidic link to ribose. The ribose, probably in the furanose form, is esterified at C3 by phosphoric acid, and the latter is presumably involved in a phosphodiester linkage of unknown nature between the individual nucleotides. As the nucleotides are present in approximately equivalent amounts, it is evident that ribonucleic acid contains four P atoms, four ribose residues and four bases per molecule. The problem remaining were the determination of the order in which the nucleotides were arranged and the nature of the labile bond which broke so readily to give rise to the nucleoides.

This formulation, which was know as the tetranucleotide hypothesis, was apparently quite well substantiated by the physical measurements made on ribonucleic acids (3) as well as by elementary analysis, and obtained such a hold on the imagination of workers in the field that it quite evidently influenced their judgment. A good example of this is given by the early enzymic work of Gulland. The use of specific phosphatases had been introduced by Takahashi (4) in Japan, with a view to finding out the types of phosphate ester linkages existing in ribonucleic acid, and Gulland and Jackson (5), working along similar lines and using a purified phosphomonoesterase preparation, found that less than 7 % of the phosphate was liberated by this enzyme. As a tetranucleotide having one free phosphomonoester group should have yielded 25 % of its phosphate as inorganic phosphate, Gulland concluded that monoesterified phosphate was absent. Had the experimental results been relied upon, it is possible that the complex nature of ribonucleic acid would have been recognised at this time. Gulland and Jackson noted during this work that some at least of the internucleotide linkages probably involved C5′, because phosphate was liberated by the joint action of phosphodiesterase and 5′-nucleotidase on ribonucleic acid. It was difficult, however, to explain at this time why all the mononucleotides produced by acid hydrolysis were nucleoside 3′-phosphates, and so Gulland was forced to postulate a migration of phosphate from C′5 to C′3 during the hydrolysis by analogy with the phosphate migration found on the hydrolysis of esters of phosphoglyceric acid (6).

The abandonment of the tetranucleotide hypothesis was gradual and followed upon a number of isolated observations. Bawden and Pirie (7) in 1937 obtained a ribonucleic acid from the tobacco mosaic virus and commented upon the fact that it was evidently of larger molecular size than was the commercial product known as yeast nucleic acid. Cohen and Stanley (8) in 1942 actually estimated the molecular weight (or more probably micellar weight) of this material as 50 000-290 000, while others (9, 10) gave estimates of the molecular weight of yeast nucleic acid as 10 000-23 000, an order of magnitude greater than expected for a tetranucleotide. Finally the development of chromatographic methods of analysis by Vischer and Chargaff (11, 12), Hotchkiss (13), and others had led to the realisation that not only are nucleic acids extremely complex substances, but that they have great variety in structure and that they normally occur as mixtures.

During the last few years considerable progress has been made in elucidating the structures involved in the ribonucleic acid molecules and while much has yet to be found out, a reasonably satisfying picture of this type of compound is beginning to emerge.

*The smaller constituents*

*The bases.* — So far only four bases have been recognized in naturally occurring ribonucleic acids, namely the purines adenine and guanine and the pyrimidines cytosine and uracil, which have all been known since the beginning of this century. Under unnatural conditions at least one other base has been found. The analogue of guanine 8-azaguanine in which C8 is replaced by N is incorporated into ribonucleic acids in a number of systems including plants, animals, bacteria and viruses, if it is present in the medium (14, 15 and unpublished), and 2-thiouracil has been reported as being incorporated into the nucleic acid of the tobacco mosaic virus (16). The former compound has been isolated in the form of the various nucleotides and dinucleotides.

The relative molecular proportions in which the bases occur in the nucleic acids from various sources are variable, but they do not differ very remarkably from unity, this probably being more a reflection of the extent to which ribonucleic acids are complex mixtures than of the composition of the individual molecules. One of the greatest divergences is shown by the well-characterised nucleic acid of the turnip yellow mosaic virus, which contains twice as many residues of cytosine as residues of guanine (17). The ratios of the bases found in nucleic acids from animal tissues also vary considerably from unity, but this may well be a reflection of the amount of degradation suffered during the extraction process. There is also ample evidence now that ribonucleic acids are very active metabolically, and so it is likely that they will vary in composition according to the metabolic state of the tissue from which they are extracted. Consequently it is mainly in sources like the purified viruses, in which the ribonucleic acid is inert, that one would expect to find consistent base ratios. Of these the tobacco mosaic virus has been very extensively investigated and the results from three different laboratories (18, 19, 20) have shown quite remarkable agreement. Figure 1 summarises some of the data on the base ratios of virus ribonucleic acids.

An attempt has been made to make generalisations about the proportions in which the various bases occur in ribonucleic acids (21) in a similar way to that proposed in the case of desoxyribonucleic acids (22), but these generalisations fall down completely when one considers the nucleic acids of the plant viruses. This may, of course, be because the latter are unusual, but so far no striking dissimilarity in their general structure or behaviour towards chemicals and enzymes has emerged, and it would seem wisest at the moment to postpone acceptance of schemes involving specific relationships between particular bases until further evidence is forthcoming. It may also be remarked that much of the published analytical data have been obtained using chromatographic methods quite unsuited to the determination of guanine, which is an extremely insoluble substance, and which requires a very acid solvent system such as that of Wyatt (23) to ensure adequate recoveries.

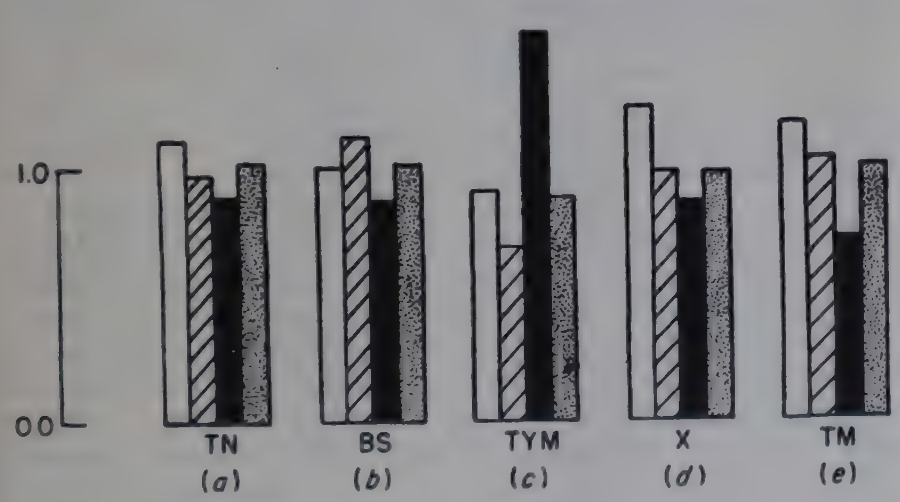


FIG. 1. — The molar proportions of the bases : adenine, guanine, cytosine, and uracil in ribonucleic acids from five plant viruses : (*a*) tobacco necrosis (bean stipple streak), (*b*) tomato bushy stunt, (*c*) turnip yellow mosaic, (*d*) an average potato virus X, and (*e*) a representative tobacco mosaic.

*The sugar.* — Kossel (24) recognised the sugar component of yeast ribonucleic acid as a pentose in 1893, and it was shown to be D-ribose by Levene and Jacobs (25) in 1909. Since that time several nucleosides have been synthesised and their structure compared with that of the natural nucleosides from yeast ribonucleic acid, and the latter have been shown to be the β-D-ribofuranosides of the purines, which are linked at N9, and the pyrimidines, which are linked at N3 (see Baddiley, 26).

Although such extensive investigation has not been carried out on other ribonucleic acids, several have been shown to contain ribose by chromatographic and other methods (17, 18, 27, 28, 29). It now seems probable from evidence obtained by enzymic degradation and chemical hydrolysis that ribose is the only sugar likely to be found in substances recognisable as 'pentose nucleic acids' so that the latter term may now be abandoned without much misgiving. In fact, as has been remarked by Brown and Todd (30), ribose has certain features which make it uniquely suitable for the function which it performs in biological systems.

*The mononucleotides.* — In 1933 Levene and Harris (31) obtained evidence, which seemed at the time to be quite conclusive, that the ribose phosphate obtained by the chemical degradation of yeast adenylic acid was ribose 3′-phosphate. Their evidence consisted in producing from inosinic acid obtained from adenylic acid by hydrolysis followed by reduction, a ribitol phosphate which was optically inactive. A similar type of degradation was also performed on guanylic acid with the same result. From this it appeared that the purine nucleotides obtained by chemical hydrolysis of ribonucleic acid were nucleoside 3′-phosphates, and it was inferred that the pyrimidine nucleotides probably had a similar structure. It was therefore with some surprise that the reports (1950) of Carter (32) and Cohn (33) were received, that the purine nucleotides were mixtures of two substances which could be separated by chromatography. It is now known that all the mononucleotides arising from ribonucleic acids by chemical hydrolysis are mixtures of two substances, first known as the *a* and *b* isomers. The true significance of these results was not evident at first, but it is now realised that they constituted a most important step in our knowledge of ribonucleic acid structure. Several types of isomerism are, of course, possible in substances as complex as nucleotides, but

Brown and Todd in 1952 (34) showed by a brilliant piece of reasoning that the most probable type of isomerism involved the phosphate group. Arguing from the known behaviour of simpler phosphate esters, they reasoned that a phosphate group at C′2 or at C′3, which was involved in a phosphodiester link, would exhibit unusual instability because of the possibility of transesterification of the phosphate to the adjacent -OH group in the ribose at C′3 or C′2, thus forming a (cyclic) 2′,3′-phosphate diester as an intermediate in the hydrolysis. Such substances should also be unstable and hydrolyse more or less at random, giving rise to a mixture of nucleoside 2′- and 3′-phosphates. Not only did this theory explain the origin of the isomeric nucleotides, and incidentally the greater stability of deoxyribonucleic acids to alkali, because they cannot form a cyclic phosphate intermediate of this type, but it showed that the position of the phosphate residues in the intact ribonucleic acids could not be deduced from a study of the products of chemical hydrolysis.

The theory advanced by Brown and Todd was soon confirmed by J. D. Smith and myself (35), by the isolation and identification of the four cyclic nucleotides which are formed by very mild alkaline hydrolysis of ribonucleic acids. The identification is relatively simple, because the cyclic mononucleotides, besides having

Fig. 2. — The electrophoretic separation of cyclic cytidylic acid (cytidine 2′ : 3′ -phosphate) and cytidine 3′ -phosphate. Electrophoresis on filter paper for 1 h. at pH 7.4, 20 V/cm. The cyclic nucleotide (left) has one less negative charge and so moves less rapidly towards the positive electrode. The white line shows where the spots were applied.

greatly different chromatographic behaviour from the other mononucleotides, lack a secondary phosphate dissociation and are therefore stable to prostate phosphomonoesterase and may, in addition, be separated from ordinary mononucleotides by electrophoresis on paper at pH 7.4, at which pH they only have one negative charge to the two charges of the latter (figure 2). Additional confirmation was obtained by comparing these compounds with synthetic cyclic mononucleotides prepared by Brown, Magrath and Todd (36), who dehydrated mononucleotides by means of trifluoroacetic anhydride. Cyclic nucleotides are also obtained by enzymic hydrolysis of ribonucleic acids and this will be described later.

The presence in ribonucleic acids of phosphate linked to C′5 of the nucleotide residues was demonstrated by Cohn and Volkin (37), who obtained some nucleoside 5′-phosphates by the action of a phosphodiesterase on partly degraded ribonucleic acid, thus confirming the earlier observations of Gulland and Jackson.

From these various observations it seemed likely, as had been anticipated earlier, that some or all of the alcoholic groups on C2′, C3′ and C5′ were involved in the internucleotide linkages, but it was by no means clear whether several types of linkage were involved or only one, or what the linkages were. The answers to these problems have come very largely from the study of the action of various enzymes.

### *The action of enzymes on ribonucleic acids*

A heat stable enzyme which hydrolysed ribonucleic acids was first (1920) found by Jones (38) in pancreas. This enzyme, ribonuclease, was crystallised in 1940 by Kunitz (39), and since that time its action on ribonucleic acids has been studied extensively. In spite of the enormous number of papers which have been published on this subject, it was almost impossible to judge what the enzyme's action was or what the products of the action were until special methods were devised to separate the latter and to analyse them. Prior to this work it was known that mononucleotides were released, that they probably consisted mainly or entirely of pyrimidine mononucleotides, and that a proportion, the 'core', comprising some 20 % of the total nucleic acid was left which had properties resembling that of the starting material, in that it was precipitated from solution by various reagents and that it did not dialyse through cellophane readily. It now seems likely that many of the apparent differences found by various workers were due to their use of a very variable material which had been subjected to harsh treatment during its isolation. It is in fact easy to demonstrate that commercial yeast nucleic acid yields a quantity of all four pairs of mononucleotides on the action of ribonuclease but more carefully prepared nucleic acid yields mainly pyrimidine *b* mononucleotides plus larger compounds.

Among this early work one observation stands out among the others and that is the report by Schmidt, Cubiles and Thannhauser (40) that ribonuclease converted all the pyrimidine nucleoside bound phosphate in ribonucleic acid into a form in which it was susceptible to phosphomonoesterase, and that therefore it was an enzyme exhibiting pyrimidine nucleotide esterase specificity. Another interesting observation which was not understandable at the time of its publication was that of Chantrenne, Linderstrøm-Lang and Vandendriessche (41), who by a dilatometric method showed that the action of ribonuclease was dual, involving a rapid reaction followed by a slower one. Both of these observations have been confirmed by subsequent work.

In 1951 we found (42) that a large part of the first-formed products of ribonuclease action on ribonucleic acids consisted of substances containing pyrimidine nucleoside residues and capable of giving rise to ordinary mononucleotides by the further action of the enzyme or the action of acid or alkali. Examination of these substances showed that they were the pyrimidine cyclic nucleotides (35), substances of the type postulated by Brown and Todd (34) as intermediates in the chemical degradation process. It was also found that the digests contained a number of small polynucleotides, most of which terminated at the phosphate end with a cyclic pyrimidine nucleotide residue or with a normal pyrimidine nucleotide residue. The former type were susceptible to further degradation by the enzyme, and the greater part of the final digest so obtained consisted of compounds of the type $n$ purine nucleotides + 1 pyrimidine nucleotide, where $n \geqslant 0$, and all these compounds had their singly esterified phosphate group attached to the terminal pyrimidine nucleoside residue (43, 44, 45). An examination of these results led to the formulation of the action of pancreatic ribonuclease as that of a phosphodiesterase specific for diesters of pyrimidine nucleoside 3′- (or 2′-) phosphates. The possibility of phosphate migration occurring during the enzyme digestion could not be excluded because of the formation of cyclic intermediates, so that the actual position of the phosphate group on the pyrimidine nucleotide residue in the original nucleic acid was still obscure, although phosphate was evidently attached at C′2 or C′3, and also at C′5, the latter because

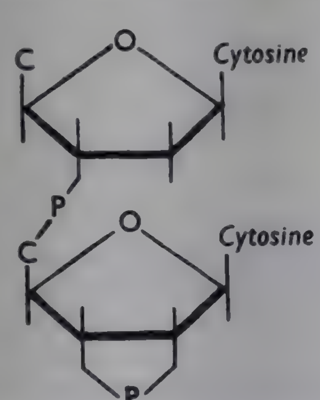


Fig. 3. — Simplified structural formula of cyclic dicytidylic acid showing that the internucleotide link must attach to C 5′ of the (lower) cyclic nucleotide residue.

dinucleotides containing a terminal cyclic nucleotide could only have the other end of the internucleotide link at this position (figure 3).

The location of the phosphate residue in the pyrimidine nucleotide internucleotide linkage was decided in an ingenious way by Brown and Todd (46), who showed that ribonuclease was able to hydrolyse synthetic pyrimidine nucleoside $b$ phosphate esters and not the corresponding $a$ esters. The position of the phosphate group in these compounds was checked in the case of the benzyl esters by removing the benzyl group by hydrogenation under conditions in which the phosphate group has no tendency to migrate.

The position of the phosphate group in the purine nucleotide residues in ribonucleic acids has been determined in two ways. By means of a phosphodiesterase from spleen, Heppel, Markham and Hilmoe (47) found that purine nucleoside $b$ phosphates were liberated from whole ribonucleic acid and from various polynucleotides without the appearance of any cyclic intermediates. Since this time it has been shown that this enzyme preparation in fact hydrolyses cyclic nucleotides to the $a$ form, an observation which quite excludes the possibility of phosphate migration having occurred (48). The other way in which the position of the phosphate group in purine internucleotide linkages was demonstrated was an empirical observation of Whitfeld and Markham (49) that it was possible to degrade dinucleotides chemically in such a way that phosphate migration was unlikely to occur. Ribonuclease resistant dinucleotides, such as adenylyl-cytidylic acid, were first dephosphorylated by means of prostate phosphomonoesterase, giving dinucleoside monophosphates (adenylyl-cytidine). The latter, having a 1,2 glycol at C2′ and C3′ of the pyrimidine nucleoside residue, are oxidised by $NaIO_4$ in neutral solution, giving a dialdehyde. The resulting compound is extremely unstable in a slightly alkaline (pH 10) medium and the purine nucleotide residue is released as the purine nucleoside $b$-phosphate (figure 4).


Fig. 4. — Degradation of adenylyl-cytidylic acid. Ultraviolet photograph of chromatogram run in 70 % isopropanol-$NH_3$. Column 1 : guanylic acid (top), adenylic acid (bottom), as reference spots. Column 2 : adenylyl-cytidylic acid. Column 3 : adenylyl-cytidine. Column 4 : periodate oxidation product of adenylyl-cytidine. Column 5 : product derived from (4) by treatment at pH 10 : adenosine 3′-phosphate (top), plus cytosine containing fragment (bottom). Column 6 : degradation product from cytidine after periodate and pH 10 treatment.

In these way it has been shown that the predominant internucleotide linkage in ribonucleic acids is of the $b$-5′ type, and it might be remarked here that no direct evidence for any other type of link has yet been obtained. The $a$ position in the mononucleotides has since been

shown by a number of methods to be the 2′ position. The most interesting of these methods, as well as the simplest, was the hydrolysis of adenylic acid *a* by means of an acid ion exchange resin to give ribose 2-phosphate (50). The adenylic acid attaches to the resin by means of the 6-$NH_2$ group of the adenine, and as soon as rupture of the glycosidic linkage takes place, the ribose 2-phosphate detaches, and so is no longer subjected to the acid medium. In this way phosphate migration is minimised. Adenosine 2′-phosphate has also been synthesised for comparison, and its structure confirmed by X-ray diffraction (51). Chromatographic behaviour and the action of several enzymes such as the *b* nucleotidase of Shuster and Kaplan (52) leads one to the conclusion that all the *b* nucleotides have their phosphate groups in the same position.

has an unusual specificity. It will break 3′-5′ internucleotide linkages at C3′ to give nucleosides phosphorylated at C5′, but is inhibited by the presence of a monoesterified phosphate residue on the C2′ or C3′ of the nucleoside (53). Because of this, it will convert a cyclic dinucleotide to a nucleoside 3′, 5′-diphosphate plus a nucleoside, the cyclic structure being broken as well (figure 6), but it will not affect a non-cyclic dinucleotide until the latter has been dephosphorylated. The products in the latter case are a nucleoside plus a nucleoside 5′-phosphate. Owing to this unique action, this enzyme should be able to give the chain sequence in a cyclic trinucleotide, which would yield on digestion a nucleoside, a nucleoside 5′-phosphate and a nucleoside 3′, 5′-diphosphate in that order from one end of the molecule.

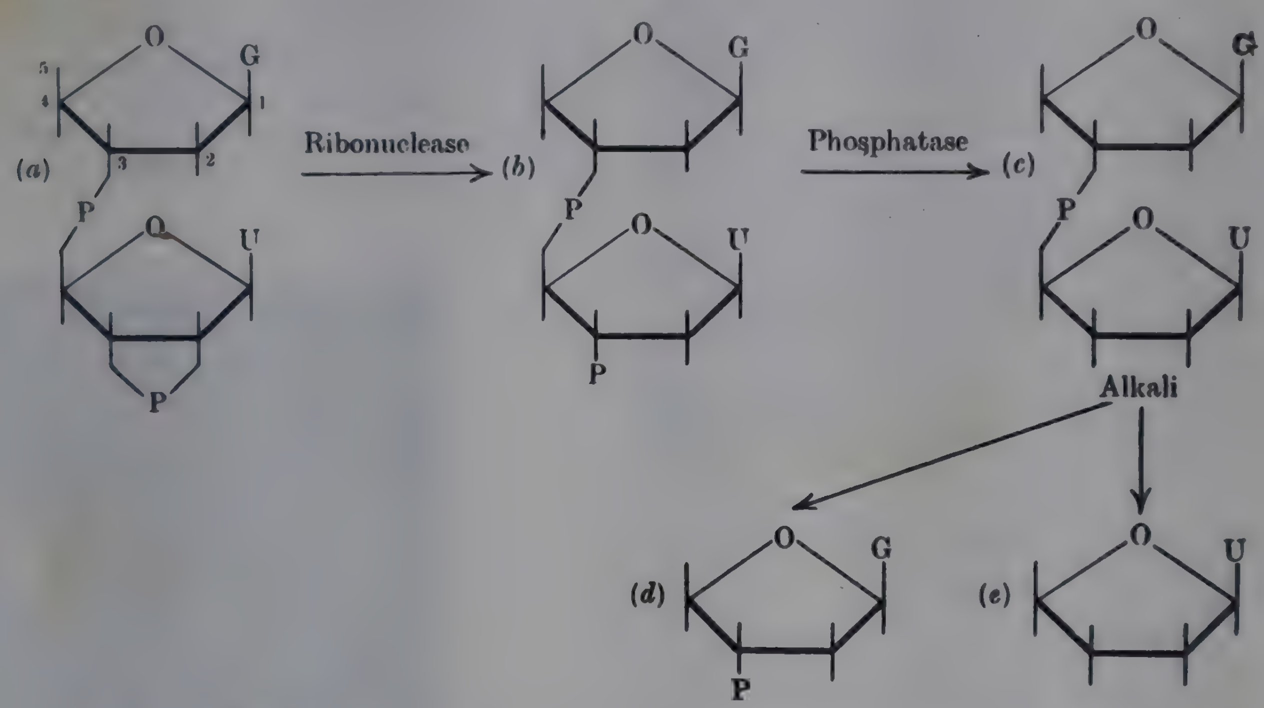


FIG. 5. — The degradation of the cyclic dinucleotide, guanylyl-cyclic uridylic acid. (*a*) The cyclic dinucleotide, (*b*) the ordinary dinucleotide, (*c*) the dinucleoside monophosphate, guanylyl-uridine, (*d*) guanosine 3′-phosphate and (*e*) uridine.

*The degradation of small polynucleotides*

It has already been mentioned that the action of ribonuclease on ribonucleic acids results in the formation of a number of small polynucleotides having certain structural similarities. Various techniques have been worked out for their investigation. The simplest one is very similar to that employed by Schmidt, Cubiles and Thannhauser (40). The terminal phosphate group is removed by phosphomonoesterase (in the case of cyclic phosphate terminations these may be hydrolysed first by 0.1 N-HCl at 20° C. to give a mixture of the 2′- and 3′-phosphates, or if they have a pyrimidine nucleotide cyclic termination, by ribonuclease), and the whole polynucleotide is hydrolysed by alkali, liberating mononucleotides plus the terminal nucleoside (figure 5). This method is of limited applicability, but may be applied to many polynucleotides including certain intact nucleic acids where it serves as a guide to the length of polynucleotide chains (45).

The phosphodiesterase of snake venom has been used with some success in degradative work. This enzyme

The phosphodiesterase from spleen has also been used for determining structure. Because of its specificity of hydrolysis cyclic dinucleotides (figure 3) yield a nucleoside 2′-phosphate from the cyclic termination and a nucleoside 3′-phosphate from the other nucleotide residue (48).

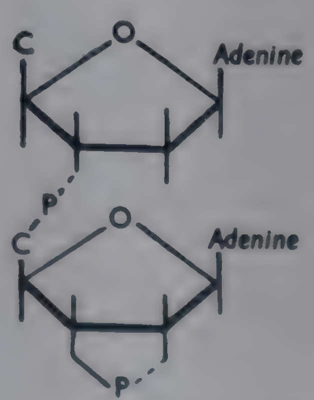


FIG. 6. — The action of snake venom phosphodiesterase on the cyclic dinucleotide cyclic diadenylic acid obtained from ribonucleic acid by mild alkaline hydrolysis. The bonds attacked by the enzyme are shown as dotted lines, and the products are adenosine and adenosine-3′,5′-diphosphate.

As has also been pointed out by Brown, Fried and Todd (54), the chemical degradation method which was used by Whitfeld and Markham (49) is potentially capable of effecting a complete stepwise degradation of polynucleotides. This method has been developed by Whitfeld (55) and has been employed successfully on a number of small polynucleotides. It is unfortunate that this quite unique procedure may never have its full potentialities realised, because so far no homogeneous polynucleotide having more than 3 or 4 subunits has been encountered, while natural ribonucleic acids appear to exist as complex mixtures.

These and similar methods have been applied to the investigation of the structure of a large number of polynucleotides produced from ribonucleic acids by enzymic hydrolysis (44, 55, 56) by acid hydrolysis (57), and by alkaline hydrolysis (58), and also on the polynucleotides produced by the synthetic action of enzymes (48). In every case only one type of internucleotide linkage has been detected, namely the 3′,5′-phosphodiester bridge.

### *The structure of ribonucleic acid chains*

As I have already mentioned, the only internucleotide linkage which has been identified with certainty is a phosphate ester linkage from C5′ to C3′ in adjacent nucleotides. Some time ago, when discussing the action of ribonuclease on ribonucleic acids, we put forward the view (45) that there was no evidence from our observations that the ribonucleic acids on which we were working were other than fairly short straight chains. This view has met with a certain amount of opposition and so I propose to review some of the data on which this view was based, together with some relevant recent work.

In general, ribonucleic acids must be isolated from tissues in active metabolism and it is exceedingly difficult to ensure that they are not degraded during the isolation procedure or that they do not combine with the nucleases present in the tissues from which they have been obtained. The latter possibility is a serious one because enzymes of this type are often very stable and exceedingly basic, so that it is possible to isolate a nucleic acid which appears to degrade itself spontaneously. There is, however, one source of ribonucleic acid from which the latter may be obtained with a fair probability of its being, if not completely native, at least as native as one is ever likely to get it, and that is from the plant viruses. A study of the two dissimilar nucleic acids from the turnip yellow mosaic virus and from the tobacco mosaic virus is rather revealing.

The nucleic acid from the turnip yellow mosaic virus has probably been investigated more thoroughly by the more recent methods than any other ribonucleic acid. It occurs as a mass having a gross molecular weight of about $2 \times 10^6$ inside a shell of protein (59) from which it may be liberated in neutral solution by means of ethanol, which denatures the protein. On the action of N-NaOH, it is converted entirely to mononucleotides showing that there is an exact equivalence of nucleosides and of phosphate joined at C2′ or C3′ (or both). On the complete action of ribonuclease, a number of cyclic adenylic and guanylic acid residues are released, which owing to the specificity of ribonuclease must have come from chain ends. These comprise about 1 in 300 of the nucleotide residues. On the action of prostate phosphomonoesterase, a certain amount of inorganic phosphate is liberated and on subsequent alkaline hydrolysis about 1 in 65 of the nucleotide residues present in the intact nucleic acid is released as a nucleoside. These results (45) have been interpreted as meaning that about 1 in 53 of the nucleotide residues have terminal 2′ or 3′ monoester phosphate groups or 2′, 3′ cyclic phosphate groups, and hence that the nucleic acid chains are about 50 nucleotides long (Markham and Smith). It has been suggested that an alternative interpretation is that the nucleic acid is branched and so has more phosphate terminations. Now, owing to the equivalence of nucleoside residues and phosphate groups, the only possible branching structure which would have the effect of increasing the number of free phosphate-bearing ends is a triple ester joining C′5 to C′3 in the main chain and attached to C′5 in the first nucleotide in the branch chain. While I consider this type of branching is unlikely it cannot be dismissed out of hand, although the general opinion is that such triple esters would be rather unstable.

The nucleic acid from the tobacco mosaic virus, unlike that which I have just discussed, when hydrolysed with N-NaOH, a step which may be accomplished without isolating it from the virus, does not give rise only to mononucleotides but also gives rise to a number of nucleosides and nucleoside diphosphates (60). This is what one would expect if the nucleic acid was built up from a string of nucleoside 5′-phosphates joined by 3′-5′ linkages. Such a structure would give a nucleoside diphosphate from one end and a nucleoside from the other. This has been confirmed by dephosphorylating the phosphomonoester groups enzymically. On subsequent alkaline hydrolysis no diphosphates are liberated, so that the latter must originate from chain ends. In this nucleic acid the chain length estimated from both ends is also about 50 residues. This means that if the nucleic acid is not a straight polynucleotide chain it must have two kinds of branches in about equal amounts, the other type of branch involved being from C2′ in the main chain to C3′ in the branch chain through a phosphate bridge. Apart from the essential improbability of such double branching, the electron micrographs of partly degraded tobacco mosaic virus show that the nucleic acid is present as a thin threadlike structure down the centre of the virus rods, in which it would be difficult to visualise a much branched structure.

While it is probably unwise to reject the possibility of chain branching in ribonucleic acids from evidence obtained on two nucleic acids obtained from plant viruses, there is a possible reason why a structure so evidently suitable for forming (hypothetical) branches should not necessarily do so. Recently it has been found that the action of pancreatic ribonuclease is reversible and that this enzyme has considerable synthetic potentialities if supplied with cyclic nucleotides as substrates (48, 61). Although this enzyme has only been isolated in bulk from pancreas, it is now evident that it is not confined to this organ (Heppel, private communication), and an enzyme having similar synthetic properties but with a purine nucleotide specificity has been isolated from

plants by Holden and Pirie (62 and unpublished). These enzymes act as transphosphorylases but are unable to form a phosphodiester link *de novo*. This type of link may, however, be preserved by the formation of a nucleoside 2′, 3′ cyclic phosphate group. From our experiments on the synthetic activities of ribonucleases, it would appear probable that they are capable of exchanging single nucleotide residues or even quite large polynucleotide chains by means of a mechanism involving the synthesis of such cyclic groupings, and this may well play an important part in the normal metabolic activity of ribonucleic acids. The presence of branches of either type would prevent this unique structure from being used to its full advantage, so I consider that unless some concrete experimental evidence of branching is forthcoming it would be unwise to regard it as a necessary feature of ribonucleic acid structure.

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# De la structure des acides ribonucléiques (échange du $^{32}P$ dans les nucléotides « a » et « b » ; comparaison des hydrolyses alcaline et enzymatique)

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(*Reçu le* 10 *août* 1955)

Deux séries de résultats personnels nous paraissent de nature à éclairer certains points encore obscurs de la structure des acides ribonucléiques.

Si l'on se réfère aux travaux de Brown et Todd, l'existence des isomères *a* et *b* des ribonucléotides s'explique par le mécanisme même de l'hydrolyse de la liaison internucléotidique : d'abord formation d'un triester phosphorique, puis hydrolyse de la liaison internucléotidique et enfin ouverture du nucléotide cyclique de deux façons différentes :

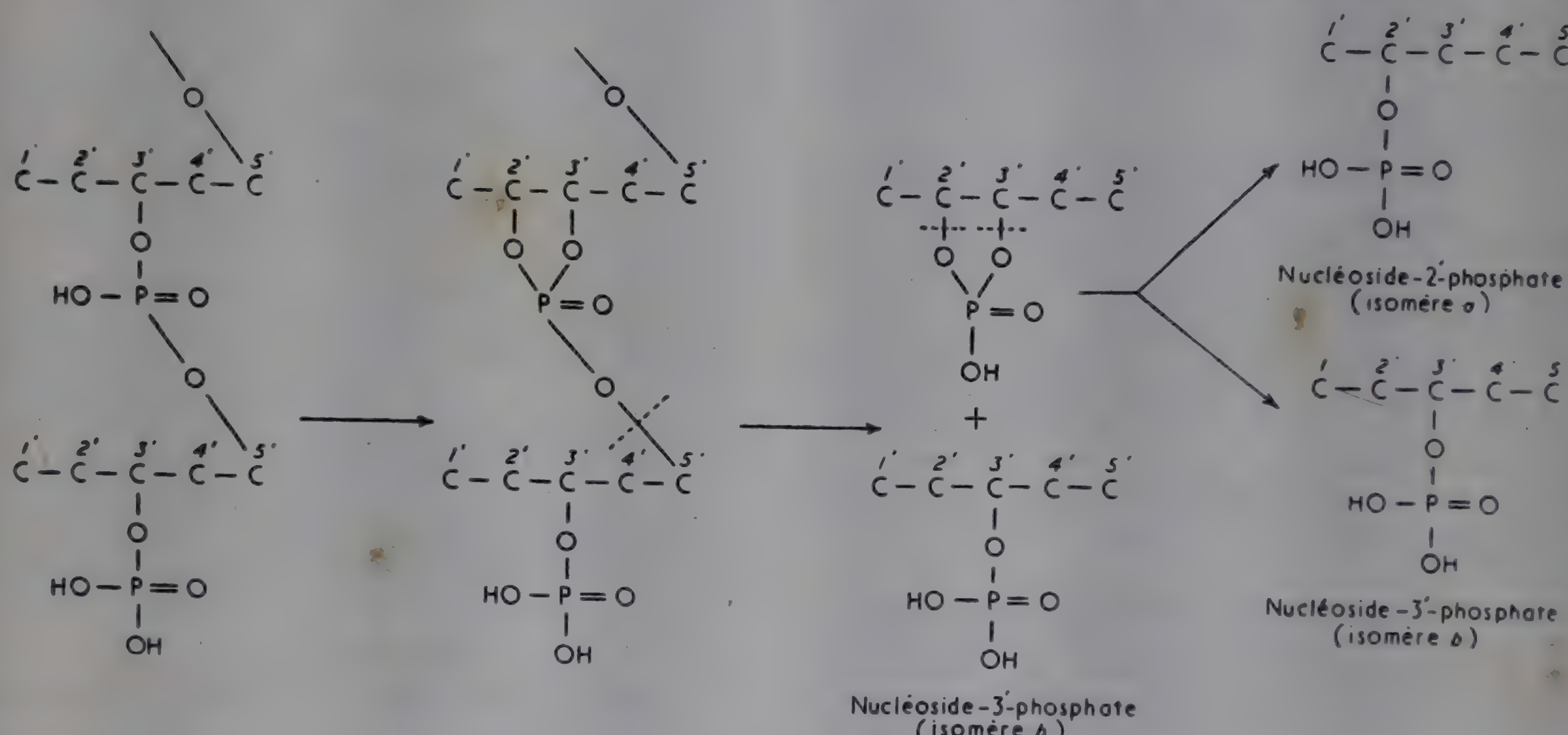


FIG. 1

Il doit donc se former, en principe, à partir des ribonucléotides situés à l'intérieur des enchaînements polynucléotidiques, des quantités égales de chaque isomère ; d'autre part, les origines des deux isomères étant identiques, la radioactivité spécifique doit être la même dans les expériences d'échange de $^{32}P$ effectuées *in vivo*. Cependant, les nucléotides terminaux doivent échapper à l'isomérisation, car leur séparation du reste de la molécule implique la formation d'un triester à partir du radical phosphoryle de l'avant-dernier nucléotide, le radical phosphoryle terminal ne subissant lui-même aucune transposition (voir schéma). On peut donc supposer que s'il y a de nombreux nucléotides terminaux, soit par suite de la brièveté des chaînes polynucléotidiques, soit à cause de l'existence de nombreuses ramifications à partir de centres phosphoriques, l'un des isomères (celui qui préexiste dans les enchaînements naturels) prédominera : comme la position terminale va presque toujours de pair avec la mobilité, il y aura une certaine probabilité pour que l'échange du $^{32}P$ soit plus actif à ce niveau et que cet isomère ait une radioactivité spécifique plus élevée.

Nous avions effectivement constaté, lors de déterminations antérieures, des différences significatives entre les radioactivités spécifiques des isomères *a* et *b* de l'acide guanylique obtenus par hydrolyse alcaline des acides ribonucléiques du tissu hépatique après injection de $^{32}P$ chez le rat (1). Mais la séparation des nucléotides *a* et *b* en chromatographie sur papier est particulièrement délicate et nous avons jugé indispensable de reprendre nos essais en suivant une technique rigoureuse qui nous mette à l'abri de toutes les causes d'erreur dans l'isolement des isomères et leur purification.

Des rats reçoivent du phosphate à $^{32}P$ en injection intrapéritonéale et sont sacrifiés après des temps très courts, les différences devant se manifester d'autant plus clairement que seuls les nucléotides les plus externes auront eu le temps d'échanger leur radical phosphoryle. Les foies sont traités suivant la technique de Schneider, puis hydrolysés par la soude. Les isomères *a* et *b* des ribonucléotides sont fractionnés suivant la technique de

Cohn et Carter et la radioactivité spécifique de chacun d'eux est déterminée. Les résultats sont rassemblés dans le tableau ci-dessous.

TABLEAU I

*Radioactivités spécifiques* (C.P.M./*mg. P*)

| | Rats sacrifiés à | | | |
|---|---|---|---|---|
| | + 5 min. | + 7 min. | + 10 min. | + 30 min. |
| Ac. cytidylique *a* | 75 | 1882 | 1818 | 37 200 |
| Ac. cytidylique *b* | 72 | 1850 | 1790 | 37 820 |
| Ac. adénylique *a* | — | 2633 | 2975 | 20 790 |
| Ac. adénylique *b* | — | 2767 | 3079 | 20 900 |
| Ac. uridylique *a* | 106 | 2365 | 3490 | 44 360 |
| Ac. uridylique *b* | 102 | 2458 | 3426 | 41 500 |
| Ac. guanylique *a* | — | 1425 | 1024 | 24 760 |
| Ac. guanylique *b* | — | 1478 | 1027 | 23 950 |

Les différences sont de l'ordre des écarts expérimentaux et l'on peut conclure que nos résultats ne sont pas en faveur de l'existence de nombreux groupes terminaux à radical phosphoryle facilement échangeable (comme l'impliquerait une structure ramifiée à partir de centres phosphoriques).

La mobilité plus grande de l'acide phosphorique de l'acide uridylique, contrastant avec l'inertie relative de l'acide guanylique, peut s'expliquer aussi bien par la spécificité de la ribonucléase, sur laquelle nous allons revenir plus loin, que par une position plus ou moins externe dans une molécule ramifiée.

Cependant, il faut admettre que nos résultats apportent un argument négatif : alors qu'une radioactivité plus grande de l'un des isomères constituerait presque une preuve de la multiplicité des groupes terminaux, l'uniformité des nombres obtenus peut s'expliquer aussi bien par la structure (nucléotides cycliques) ou le comportement biochimique de ces groupes terminaux, que par leur rareté.

L'étude des produits de la dégradation ribonucléasique des acides ribonucléiques d'une part, la détermination des groupements terminaux d'autre part, ont conduit Markham et Smith à proposer une représentation linéaire de la molécule des acides ribonucléiques. La nature des produits d'hydrolyse ribonucléasique permet de considérer la ribonucléase comme une pyrimidine-nucléoside-phosphodiestérase et cette spécificité explique la libération préférentielle des nucléotides pyrimidiques (sous forme de mono- ou d'oligonucléotides) sans faire appel à une structure ramifiée dans laquelle les chaînes latérales auraient une composition à prédominance nettement pyrimidique.

Cependant, il faut insister sur le fait que l'étude cinétique de l'action d'agents chimiques d'hydrolyse (soude, ammoniaque) fournit des résultats qui se rapprochent beaucoup de ceux de l'hydrolyse ribonucléasique. Des hydrolyses sont réalisées à température ordinaire par la soude 0.1 N ou 0.01 N ou par l'ammoniaque à 20 % ( 4 %de $NH_3$). Des prélèvements sont effectués à intervalles réguliers : toutes les 15 secondes au cours des hydrolyses sodiques, toutes les heures au cours des hydrolyses ammoniacales. L'acidification des hydrolysats par l'acide formique permet d'éliminer le « noyau » par centrifugation.

La chromatographie directe de la solution surnageante dans le système-solvant *iso*propanol : eau (atmosphère $NH_3$) de Markham et Smith révèle sa richesse en oligonucléotides.

Si l'on soumet ceux-ci à une hydrolyse sodique totale, la chromatographie montre dans les premiers prélèvements la prédominance considérable de l'acide uridylique : par exemple, au temps + 30 min. de l'hydrolyse ammoniacale, les proportions des acides uridylique, guanylique, cytidylique et adénylique sont respectivement : 20 : 1 : 2 : 2.

La libération préférentielle des nucléotides pyrimidiques qui, dans le cas de la ribonucléase, pouvait s'expliquer par la spécificité de l'enzyme, se conçoit plus difficilement dans le cas d'agents purement chimiques. Rappelons que c'est une expérimentation analogue qui avait conduit Chargaff à proposer une structure ramifiée des acides ribonucléiques. Aussi, une étude comparative plus poussée des produits d'hydrolyse ribonucléasique et alcaline mérite-t-elle d'être entreprise ; elle devrait fournir des indications précieuses concernant la nature des points de fragilité de la molécule et l'existence possible de ramifications.

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## Concerning branching in ribonucleic acids

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(*Received* 8 *October* 1955)

The fact that nucleoside diphosphates and nucleosides were produced from RNA in relatively large and equivalent quantities by the action of a diesterase from snake venom was at first interpreted by Cohn and Volkin (1) in terms of branching through triply esterified ribose, a possibility first envisioned by Brown and Todd (2). Supporting this interpretation were the observations that (*a*) the amounts were too large to come only from the ends of unbranched chains, unless these were of the order of size of decanucleotides, (*b*) the action of diesterase upon RNA previously dephosphorylated with bone phosphatase gave no less a quantity of pyri-

midine nucleoside diphosphates than untreated RNA, (*c*) the rate of action of the diesterase upon polynucleotides with free phosphate end groups was negligible.

While branches from pyrimidine nucleoside to pyrimidine nucleoside offered a ready explanation of these observations, subsequent observations and considerations have undermined the validity of the argument. Evidence is accumulating to show that RNA as used in such experiments may be enzymatically partially degraded by nucleases present in itself (3), in the diesterase preparation, or as contaminants in the apparatus used (4), and may therefore actually exist as shorter units (*e.g.*, decanucleotides) at the moment of diesterase attack. Furthermore, nuclease degradation proceeds *via* cyclic pyrimidine nucleoside phosphates (5), which are readily attacked by the diesterase and which would yield ultimately the mixture of 2′, 5′ and 3′, 5′ pyrimidine nucleoside diphosphates found (1).

Recent data (6, 7) indicate a wide variability in the amounts of the diphosphates formed, consistent with differences in nucleolysis, among RNA's. A variable tendency of RNA's in neutral solution to undergo seemingly spontaneous degradation has been observed (6). The appearance in such degradation mixtures of a few products of the ribonuclease produced type, and the inhibition of this process by drastic treatment which would destroy the very stable pancreatic ribonuclease, lend support to the nuclease contaminant hypothesis of large diphosphate production.

The high probability that RNA preparations, particularly from mammalian tissue, contain or have been partially degraded with nucleases, or that the enzyme mixtures as used contain them, makes it impossible to depend upon the production of nucleoside diphosphate as evidence of branching.

However it should not be inferred that the likelihood of nuclease contamination within the RNA itself, or even its partial demonstration in a case or two, is sufficient to conclude that all previous preparations are similarly contaminated or degraded, nor that drastic treatment (3), sufficient to destroy pancreatic ribonuclease, is required in order to be assured of an undegraded RNA sample. Some tissues, yeast being an example, are not particularly rich in nuclease activity, nor are their nucleases necessarily as resistant as pancreatic ribonuclease. While one preparation of yeast RNA did yield large amounts of diphosphates with venom (1), other preparations have yielded amounts as small as those obtained from yeast RNA prepared in the more drastic manner (7).

The largest yield of diphosphates thus far demonstrated has been obtained on a sample of calf-liver RNA (1). This same preparation, when subjected to methanolysis, yields number and kinds of end groups which are consistent with the numbers and kinds of diphosphates obtained from the venom digests of undegraded RNA's (*ca.* 2 %) (8). From this it may be inferred that if degradation of this calf-liver RNA was responsible for the high diphosphate yield, it took place after solution of the dried preparation, not during its original isolation, and is therefore due to a self-contained enzyme. A counter argument to this hypothesis is the inability of the methanolysis procedure to detect cyclic end groups which might have been produced by prior nuclease action. However these, if present, should permit a faster rate of diesterase action than is actually observed (9).

With regard to branching through phosphate, the chemical evidence seems to indicate that phosphate triesters are sufficiently unstable in neutral aqueous media to hydrolyse during most methods of RNA preparation (10). No further evidence is available to show the presence or absence of such branches. The occurrence of pyrimidine 3′ nucleotides in diesterase digests can no longer be considered as evidence in view of the arguments for nuclease contamination in RNA's or in diesterases presented above. The same considerations hold for phosphatase analysis of end groups (1, 11); nuclease contamination could give an indefinite number of end groups.

Thus there seems to be a complete lack of definitive evidence for branching and a degree of support for the concept of RNA preparations as unbranched chains of about 50-200 nucleotides in length.

However, the absence of positive evidence for branching cannot be construed as eliminating the possibility of either type. Preparative treatments may eliminate phosphotriesters. Chemical or enzymic degradations have also their limitations. Some different or more definitive approach is needed before the question of branching can be resolved.

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# The uridine coenzymes

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(*Received* 5 *August* 1955)

Adenylic, guanylic, cytidylic and uridylic acids have been known for a long time as nucleic acid constituents but the occurrence of these four nucleotides in smaller molecules is a more recent discovery. In 1925 Hoffman (1) obtained crystalline adenylic acid from pig blood and in 1927 it was found in muscle by Embden and Zimmermann (2). Subsequently through the effort of many workers and specially of Warburg, Euler and Lohmann, several combined forms of adenosine-5-phosphate (AMP) were discovered and found to act as coenzymes in many metabolic processes.

None of the other three nucleotides had been found to occur in biological material either free or forming part of small molecules. In 1950 uridine-5′-phosphate containing compounds started to be isolated and at such a rate that the number of these substances which are now known is about the same as that of the adenylic acid series (*).

Some substances containing guanosine-5′-phosphate and cytidine-5′-phosphate have also been discovered so that adenylic acid has lost the unique position, as a building block of coenzymes, which it was believed to have. It now appears that any of the nucleotides which are present in ribonucleic acids namely adenylic, guanylic, cytidylic and uridylic acids may have a coenzyme form so that there probably exists a functional relationship between coenzymes and nucleic acids, the nature of which will surely be elucidated in the next few years.

Many enzymes acting on uridine compounds are now known and their action will be reviewed here in the following order :

(*i*) processes leading to the synthesis of uridine-5′-phosphate, (*ii*) phosphorylation of UMP5′ to the di- and triphosphates, (*iii*) synthesis of uridine diphosphate sugar compounds, (*iv*) uridine diphosphate glucose and its role in metabolism, (*v*) uridine diphosphate glucuronic acid and glucuronide synthesis, (*vi*) uridine diphosphate hexosamine compounds.

### *The biosynthesis of uridine-5′-monophosphate*

The presence of UMP5′ in the soluble nucleotides of cells was disclosed when it was identified as a product of acid or alkaline hydrolysis of UDPG (3), and when it was found in yeast extracts (4). Subsequent studies revealed the presence of free UMP5′, UDP and UTP in liver, in tumors (5, 6, 7) in certain commercial fermentation mixtures (8), in yeast (9) and in muscle (10).

Many of the enzymic steps which lead to the formation of UMP5′ have been worked out by Lieberman and Kornberg and may be summarized as shown in figure 1.

Reactions (*1*) and (*2*) leading from ureido succinic acid to dihydroorotic acid (11) were studied with enzymes obtained from anaerobic bacteria isolated using a selective medium containing orotic acid as source of carbon.

Reaction (*3*), that is the formation of orotidine-5′-phosphate from orotic acid and 1-pyrophopho-5-phosphoribose (12) was an outstanding discovery since it disclosed a new mechanism for the introduction of the 5-phosphoribose moiety. These experiments were carried out with enzymes from yeast and pigeon liver. The following step is the decarboxilation of orotidine-5′-phosphate to yield uridine-5′-phosphate (13).

The work which has been carried out in intact animal cells using labelled substrates is consistent with the scheme presented in figure 1 as is also that of Hulbert and Reichard (14) who used labelled orotic acid and soluble liver enzymes.

Some enzymes acting on uridine have also been described such as phosphorylases (15, 16) and hydrolases (17).

### *The phosphorylation of uridine-5′-monophosphate*

Several enzymes concerned with the phosphorylation and dephosphorylation of uridine phosphates have been studied.

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(*) Abbreviations used : UMP for uridine-5′-monophosphate, UDP for uridine-5′-diphosphate, UTP for uridine-5′-triphosphate, UDPG for uridine-diphosphate-glucose, UDPgal for uridine-diphosphate-galactose, UDPag for uridine-diphosphate-acetyl-glucosamine ; P for orthophosphate, PP for pyrophosphate.

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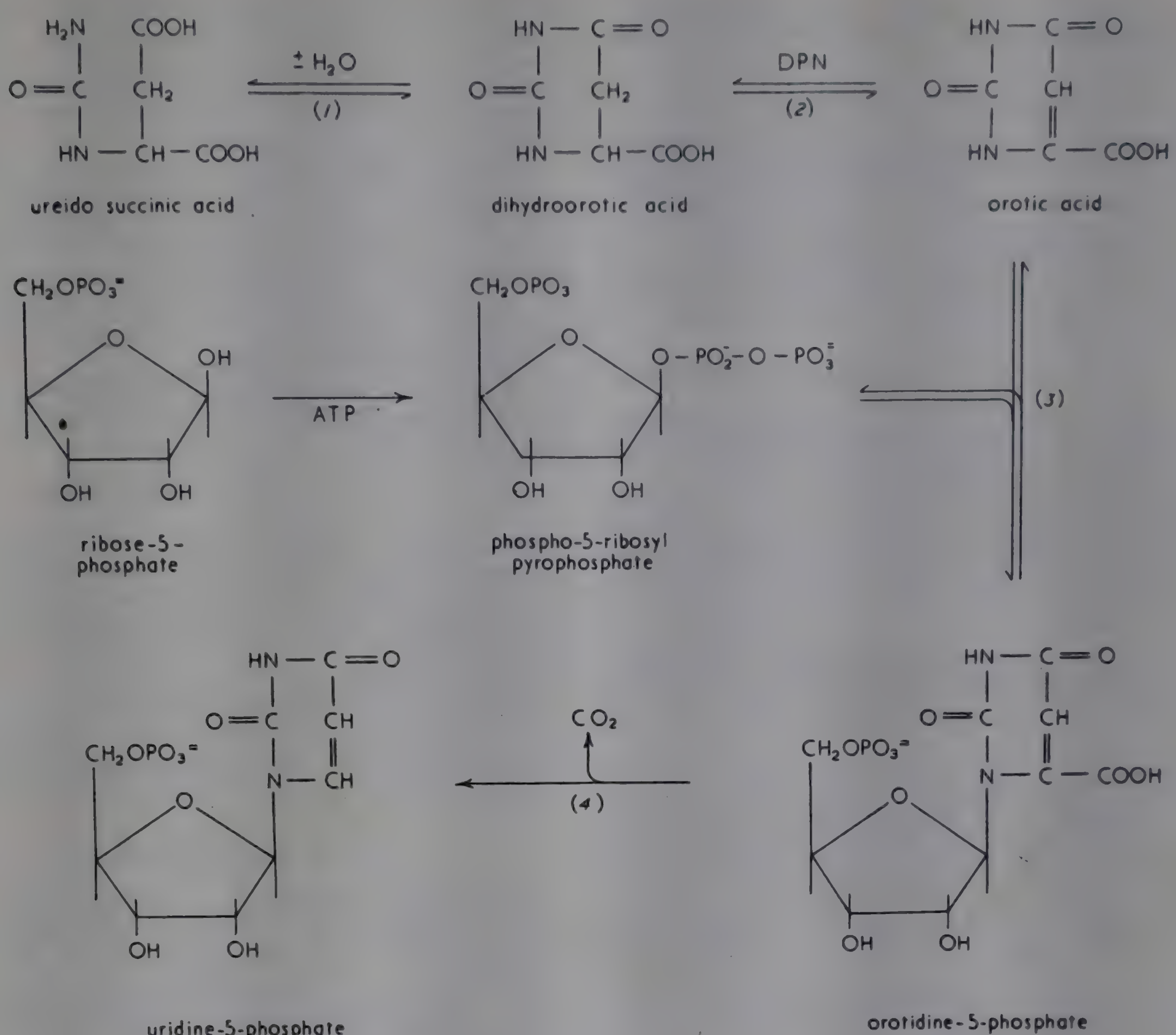



**Fig. 1**

The introduction of a phosphate group into UMP to yield UDP is analogous to the formation of adenosine diphosphate (ADP) from adenosine monophosphate (AMP). In the latter case such a step is catalysed by adenylate-kinase (myokinase) as follows :

$$AMP + ATP \rightleftarrows 2\,ADP \quad (5)$$

According to Lieberman, Kornberg and Simms (18) the same enzyme may catalyze a reaction involving uridine phosphates :

$$UMP + UTP \leftrightarrows 2\,UDP \quad (6)$$

and also a mixed reaction :

$$ATP + UMP \rightleftarrows ADP + UDP \quad (7)$$

The specificity of the enzymes has not been studied in detail. Strominger, Heppel, Maxwell (19) and also Munch-Petersen (20) seem to be in favour of the mixed formulation and suggest the name of nucleoside monophosphate kinase for the enzyme.

As to the formation of UTP from UDP it has been studied by Berg and Joklik (21, 22). They purified enzymes from muscle and yeast which catalyze the reaction :

$$ATP + UDP \rightleftarrows ADP + UTP \quad (8)$$

The evidence indicated that the same enzyme catalizes the phosphorylation of inosine diphosphate to the triphosphate and therefore it was named nucleoside diphosphate kinase. This name has been abbreviated to 'nudiki' by Kalckar and to be consistent one could abbreviate the name of the enzyme corresponding to reaction (7) to 'numoki'. Thus the joint action of 'numoki' and 'nudiki' would explain the formation of UTP from UMP.

Herbert, Potter and Yasuyuki (23) have studied the distribution of these enzymes in different cell fractions. The mitochondria were found to convert UDP to UTP, as if they contained 'nudiki'. The supernatant obtained after centrifuging off the mitochondria phosphorylates UMP to UDP that is as if 'numoki' were present.

Another mechanism by which UDP can be phosphorylated was described by Kornberg (24) and is the following :

$$\text{Phosphopyruvate} + \text{UDP} \rightleftarrows \text{pyruvate} + \text{UTP} \quad (9)$$

This reaction is catalyzed by phosphopyruvate kinase and it has been reported (25) that the rate with the different diphosphates is as follows : adenosine diphosphate, 100; guanosine diphosphate, 19; inosine diphosphate, 12; uridine diphosphate, 3 and cytidine diphosphate, 2.

Several reactions in which UTP acts as phosphate donor have been described. Thus Kornberg (24) observed the reaction :

$$\text{UTP} + \text{glucose} \rightleftarrows \text{UDP} + \text{glucose-6-phosphate} \quad (10)$$

It has been suggested that this is a complex reaction in which ATP is the donor and that the enzymes involved are 'nudiki' and hexokinase (21, 26).

The phosphorylation of creatine by UTP is also considered to be indirect. However in a similar case, in the phosphorylation of fructose-6-phosphate, Ling and Lardy (27) found that UTP acts directly as follows :

$$\text{UTP} + \text{fructose-6-P} \rightarrow \text{UDP} + \text{fructose diphosphate} \quad (11)$$

The reaction was studied with highly purified reactants and enzymes and the rate was found to be nearly the same whether the phosphorylant was ATP, UTP or ITP.

Some other enzyme reactions which appear to be more or less specific for uridine phosphates have been described. For instance a dephosphorylation of UDP is brought about by an enzyme which has been obtained from calf liver acetone powder (19). The release of phosphate from UDP was found to be about 100 times faster than from UTP, ADP or ATP. In combination with 'numoki' it hydrolized UTP 50 times faster than ATP.

A similar enzyme obtained from pork kidney has been found (28) to dephosphorylate the different diphosphates at the following rates : IDP, 36; GDP, 28; UDP, 24; ADP, 0.7. Kalckar (29) has also reported that crystalline myosine catalyses a dephosphorylation of UTP which is 3-6 times faster than that of ATP.

### *The biosynthesis of UDP-sugar compounds*

A general mechanism by which UDP-sugar compounds may be synthesized enzymically is :

$$\text{UTP} + \text{sugar-1-phosphate} \rightleftarrows \text{UDP-sugar} + \text{inorganic pyrophosphate} \quad (12)$$

Such a process was suggested by Trucco (30) as an explanation for the fact that on incubating G-1-P, UDP and ATP with yeast extract a synthesis of UDPG was observable.

The reaction was first studied directly by Kalckar and coworkers (26, 31) going from right to left with uridine-diphosphate-glucose (UDPG) as follows :

$$\text{UDPG} + \text{PP} \rightleftarrows \text{UTP} + \text{G-1-P} \quad (13)$$

A powerful pyrophosphorylase was obtained from Lebedew juice treated as described by Warburg for the preparation of *Zwischenferment* and further purified by precipitation with ammonium sulfate between 0.6 and 0.7 saturation. The test consisted in adding the reactants, phosphoglucomutase and TPN. The glucose-1-phosphate (G-1-P) formed gives glucose-6-phosphate (G-6-P) which reduces TPN so that the reaction can be followed spectrophotometrically.

The correctness of the fomulation was checked by chromatographic methods using radioactive inorganic pyrophosphate. The reaction is reversible with an equilibrium constant of about one. The enzyme does not function if β-glucose-1-phosphate is added instead of α-glucose-1-phosphate (32) and it is also inactive with UDP-acetyl-glucosamine (UDPag) or UDP-galactose (UDPgal) instead of UDPG.

An enzyme acting on UDP-acetylglucosamine has been found in liver nuclei (33) and also in crude yeast extracts. The reaction is :

$$\text{UDPag} + \text{PP} \rightleftarrows \text{UTP} + \text{Pag} \quad (14)$$

The search for the enzyme in nuclei was suggested by the work of Hogeboom and Schneider (34) on DPN pyrophosphorylase. Nuclei also contain UDPG pyrophosphorylase (35) but have no activity on UDP-glucuronic acid. As to UDP-acetylgalactosamine it has not been studied.

Some indirect evidence for the presence of a UDPgal pyrophosphorylase in extracts of *Saccharomyces fragilis* has been presented (36) but the existence of such an enzyme remains to be proved by direct methods.

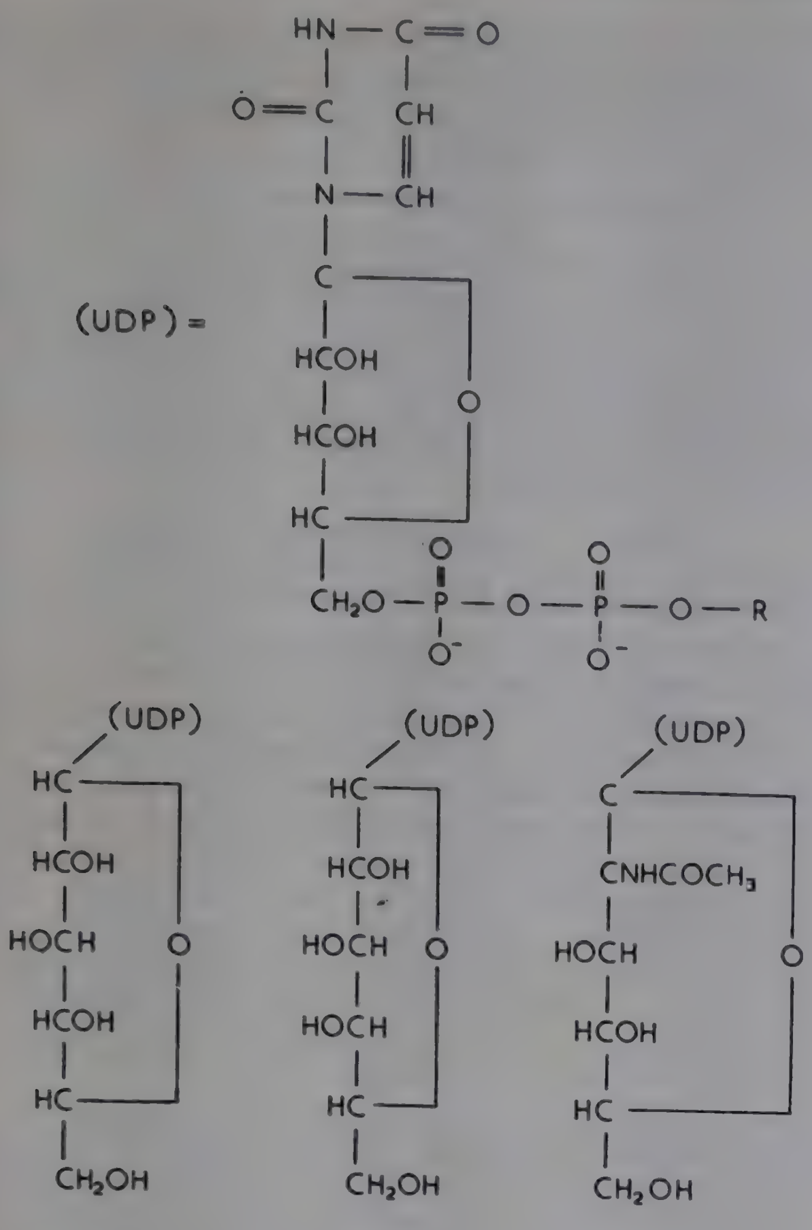
### *Uridine diphosphate glucose*

*Structure and properties.* — The structure of UDPG shown in figure 2 can be considered to be established beyond doubt. Treatment with acid at pH 2 during 10 min. at 100° C. leads to the formation of glucose and UDP. With stronger acid (N during 15 min. at 100° C.) one phosphate group of UDP is hydrolyzed leaving UMP 5′ (3).

The alkaline type of hydrolysis leads to UMP5′ and cyclic-glucose 1, 2-monophosphate which on further hydrolysis yields a mixture of about 25 % glucose-1-phosphate and 75 % glucose-2-phosphate (4). This type of hydrolysis seems to be the main cause of trouble in the purification of UDPG since the principal contaminant in the final preparations is usually UMP and not UDP.

The formation of a cyclic glucose ester from UDPG indicates that the glucose rest is joined by an α linkage since in this configuration the hydroxyls at positions 1 and 2 are *cis* so that the 1, 2 ester can be formed. In UDPag the hydroxyl at position 2 is absent and this substance is more resistant to alkali than UDPG, as is also GDPM in which the 1 and 2 hydroxyls are *trans*.

Confirmation on the α structure of the glucose residue in UDPG was obtained by polarimetric measurements (4). The decrease in dextro-rotation produced by mild acid hydrolysis of UDPG was comparable with that obtained on hydrolysis of glucose-1-phosphate. Still further evidence is the previously mentioned fact that UDPG pyrophosphorylase is specific for α glucose-1-phosphate (33).



UDPG UDPgal UDPag

Fig. 2

Other findings which led to the establishment of the structure of UDPG were : the fact that electrometric titration shows two primary acid groups and no secondary and that a nucleotide pyrophosphatase leads to the liberation of glucose-1-phosphate.

UDPG was first obtained from yeast (3, 37) and has since been shown to occur in every organism investigated. It is present in plants (38, 39, 40) in mammalian liver (33, 41) and in tumors (5).

*Chemical synthesis.* — The synthesis of UDPG has been an outstanding achievement of Todd and coworkers (42) who have also developed general methods for the synthesis of similar compounds.

The two halves of the UDPG molecule were joined to form the pyrophosphate bond as follows. The pyridine salts of G-1-P and UMP5′ were mixed with dicyclohexylcarbodiimide ($C_6H_{11}-N=C=N-C_6H_{11}$). The products of this reaction were G-1-P, UMP5′, UDPG and Di(UMP5′) pyrophosphate. The yield in UDPG was rather low (3.5 %) but preparations of 40 % purity could be obtained by suitable purification. The failure to obtain more UDPG seems to be due to the fact that it is decomposed during the reaction to give cyclic glucose-1, 2-monophosphate and UMP5′.

Moreover two methods have been described for the synthesis of UDP (43, 44) and more recently Hall and Khorana (45) developed a simple and elegant method for UDP and UTP using also dicyclohexylcarbodiimide.

*Galactowaldenase.* — The mechanism by which UDPG acts as a coenzyme in the transformation of galactose-1-phosphate (gal-1-P) into glucose-1-phosphate (G-1-P) has been formulated as follows :

$$\text{UDPG} + \text{gal-1-P} \rightleftarrows \text{UDP-gal} + \text{G-1-P} \qquad (15)$$

$$\text{UDP-gal} \rightleftarrows \text{UDPG} \qquad (16)$$

$$\text{Sum :} \quad \text{gal-1-P} \rightleftarrows \text{G-1-P} \qquad (17)$$

The supporting evidence consists in the fact that reaction (*16*) could be detected in the backward direction : on incubating UDPG with extracts of galactose adapted yeast there was a partial conversion to UDPgal (46). Moreover, Trucco (47) found that on incubating UDPG and $^{14}C$ labelled glucose-6-phosphate the label became distributed between these two compounds. These experiments were carried out with crude extracts which contained phosphoglucomutase so that there was a rapid conversion of glucose-6-phosphate into glucose-1-phosphate. Furthermore Kalckar and coworkers (48) observed an exchange of the labelled P of glucose-1-phosphate with that of UDPG. Some studies on the purification of the enzyme system have been reported (49) and Maxwell and Kalckar (unpublished) have found that on heating the extracts to 60° C. at pH 5 the enzyme catalysing reaction (*16*) is destroyed whereas (*15*) persists.

The equilibrium of the reaction gal-1-P to G-1-P is reached when the concentrations are about 25 and 75 % respectively (50). These results were obtained starting with known mixtures of G-1-P and Gal-1-P in extracts where phosphoglucomutase was inhibited with arsenate. Hansen and Craine (51) have obtained similar values (21-27 % of gal-1P) using labelled substrates and an enzyme from *Lactobacillus* sp. It may be mentioned that the extent to which UDPG is transformed into UDPgal is also about 25 % (46).

These studies have left unexplored the most interesting part of the reaction, that is, the mechanism by which the hydroxyl at position 4 becomes inverted. Some of the mechanism by which can be considered are :

(*i*) a direct inversion ; (*ii*) oxidation to produce a dienol followed by reduction ; (*iii*) loss of water to give a double bond ; (*iv*) a $C_3$-$C_4$ split to give a primary alcohol and aldehyde group. Some of these possible mechanism have been discussed elsewhere (52, 53) but no experiments have been reported.

UDPgal which was first obtained by enzymic action on UDPG (46) has been found preformed in liver (41) and in yeast (9).

*Galactose metabolism.* — The utilization of galactose in yeast (54) and in animal tissues (55) starts with a phosphorylation to galactose-1-phosphate, which is then transformed to glucose-1-phosphate by galactowaldenase. Most of the studies on galactowaldenase have been carried out with yeast but there is evidence that the same mechanism is used in animal tissues. Thus liver contains both UDPG and UDPgal and the enzymes which

catalyse reaction (*15*) and (*16*) have been found in liver extracts by Kalckar and Maxwell (unpublished). In galactosaemia which consists in a specific inability to metabolize galactose normally there may be a defect in galactowaldenase. Thus Schwarz, Goldberg, Komrower and Holzel (56) found that in galactosaemic patients there occurs an accumulation of gal-1-P in blood after feeding galactose.

The problem of the mechanism of lactose synthesis in the mammary gland has resisted elucidation despite the considerable effort which has been expended on it. It is known that the lactose moiety arises from glucose directly, that is without breakdown and resynthesis. UDPG has been found in lactating mammary gland by enzymic methods (57, 58) and by column chromatography (59). According to Smith and Mills (59) it is about 3 times more abundant in the mammary gland that in the liver of the same animal. According to the results obtained by Kittinger and Reithel (60), the glucose part of lactose arises from glycogen and the galactose moiety from glucose-1-phosphate, but the intimate mechanism of the reaction remains unknown.

*The synthesis of trehalose phosphate.* — Trehalose is known to be a constituent of certain plants, fungi and yeast. Its physiological role is obscure and it is usually considered as the reserve substance of molds as sucrose is considered a reserve substance in plants.

Several years ago Robison and Morgan (61, 62) in the course of their brilliant studies on sugar phosphates isolated trehalose phosphate. This was a great feat at that time when the methods of isolation were not what they are today. Few other studies were reported on trehalose phosphate until it was found that the disappearance of UDPG in yeast extracts is greatly increased by the presence of glucose-6-phosphate (63). Further investigation led to the identification of trehalose phosphate as the product. The reaction may be written as follows :

$$\text{UDPG} + \text{G-6-P} = \text{UDP} + \text{trehalose phosphate} \quad (18)$$

The decrease in reducing power (of G-6-P) and of labile glucose (UDPG) compared well with the amount of UDP formed. The reaction product was identified by paper chromatography and electrophoresis before and after treatment with phosphatase. As to the further metabolism of trehalose phosphate nothing has been found except its hydrolysis with phosphatase to give the free disaccharide. In trehalose the glucose is joined in an $\alpha$ linkage like in sucrose and both of the disaccharides are non reducing. In view of the manifold reactions which lead from sucrose to polysaccharides an attractive hypothesis is to assume that trehalose is also used in such a type of synthesis and that in yeast the cellulose-like cell wall material, glucan might arise from trehalose.

*Sucrose synthesis.* — During an investigation on the mechanism of $CO_2$ fixation in green plants Buchanan *et al.* (64) observed that labelled $CO_2$ became rapidly incorporated into the nucleotide fraction. From this fraction, radioactive glucose, galactose and mannose could be obtained by mild acid hydrolysis while after alkaline treatment the products were phosphate esters behaving chromatographically like cyclic glucose-1, 2-monophosphate. This evidence for the presence of UDPG and UDPgal in green plants was correlated with the presence of sucrose phosphate which also becomes labelled rapidly (64, 65) and it was suggested that the following reaction occurred :

$$\text{UDPG} + \text{Fructose-1-P} \rightarrow \text{UDP} + \text{sucrose phosphate} \quad (19)$$

The sucrose phosphate was believed to have the phosphate in position 1 of fructose because paper chromatography of the hydrolysate showed spots of glucose and of a fructose ester different from fructose-6-phosphate.

Enzymes which lead to the synthesis of sucrose have been obtained from wheat germ (39, 66, 67). They catalyse the reactions :

$$\text{UDPG} + \text{Fructose} \rightleftarrows \text{UDP} + \text{sucrose} \quad (20)$$

$$\text{UDPG} + \text{Fructose-6-P} + \text{UDP} \rightarrow \text{sucrose phosphate} \quad (21)$$

The enzymes could not be separated completely but sufficient evidence was obtained to conclude that two different enzymes are involved. The enzyme catalysing the formation of free sucrose (reaction *20*) was detected in many different materials of plant origin thus proving its widespread occurrence. As to the sucrose phosphate formed in reaction (*21*) it was found to give glucose and fructose phosphate by mild acid hydrolysis. The fructose ester was identified as the 6 phosphate by its rate of hydrolysis in acid and in alkali and by its enzymic conversion to glucose-6-phosphate. The product is therefore different from the one postulated by Buchanan (65).

These experiments have clarified the mechanism of sucrose synthesis in plants. On the basis of previous data it had been concluded that phosphoric esters are involved in this synthesis. Moreover an enzyme, sucrose phosphorylase, (68) had been isolated from bacteria (*Pseudomonas saccharophila*) which catalyses the reaction :

$$\text{Sucrose} + \text{P} \rightleftarrows \text{G-1-P} + \text{fructose} \quad (22)$$

Since the reaction is reversible it has been possible to prepare sucrose starting with glucose-1-phosphate and fructose. However the position of the equilibrium is not in favour of the synthesis in contrast to the reaction in which UDPG is involved. The equilibrium values are :

$$\frac{\text{Sucrose} \times \text{P}}{\text{G-1-P} \times \text{fructose}} = 0.05 \qquad \Delta F = +\,1700 \text{ cal.}$$

$$\frac{\text{Sucrose} \times \text{UDP}}{\text{UDPG} \times \text{fructose}} = \text{appr. } 5 \qquad \Delta F = -\,1000 \text{ cal.}$$

Accurate values for the equilibrium of the UDPG enzyme have not been obtained but the direction in which the equilibrium is displaced seems to be consistent with the physiological role of the enzymes. Sucrose phosphorylase is probably used in the catabolism of sucrose by *P. saccharophila*, whereas the UDPG enzyme appears to be used by plants for the synthesis of sucrose.

No data has been obtained for the equilibrium of the reaction catalysed by the enzyme which acts on UDPG and fructose phosphate nor on the distribution of the activity in different plant materials. Perhaps in some cases sucrose is formed predominantly from fructose-6-phosphate and in other from free fructose. Such a

difference in the pathway might explain the fact that infiltration of radioactive glucose gives sucrose predominantly labelled in the glucose in apple tissue, (69) while with canna indica (70), sunflower (71) or tobacco (72) leaves the label appears about equally in both halves of the sucrose. The explanation might be that in the apple, reaction (*20*) takes place so that the fructose half of sucrose would arise from the free fructose pool and would have a low radioactivity. In the canna or sunflowers leaves, reaction (*21*) would be predominant so that both parts of the sucrose would arise from the hexosephosphate pool. The sucrose phosphate formed would be equally radioactive in both halves and would be converted to sucrose by phosphatase action.

Turner (73, 74) obtained an increase in sucrose incubating a pea extract with glucose-1-phosphate. In this case it is likely that reaction (*20*) or (*21*) involved plus other enzymes. UDPG pyrophosphorylase would be necessary for the synthesis of UDPG and also the enzymes for the series glucose-1-phosphate → glucose-6-phosphate → fructose-6-phosphate → fructose. In addition there should be a phosphorylating system for regenerating UTP from UDP. Cardini (unpublished) obtained a small synthesis of sucrose with a dialysed pea extract plus ATP, glucose-1-phosphate and catalytic amounts of UDPG. No sucrose was formed on omitting any of these substances.

### *UDP-glucuronic acid and glucuronide synthesis*

In the course of their study on glucuronide synthesis, Dutton and Storey (75, 76) observed that liver extracts were inactive unless a heat stable fraction of liver was added. They were able to purify the active substance up to the point where it could be concluded that it was UDP-glucuronic acid (UDPguc). On incubating this substance with a liver homogenate and aminophenol or menthol about one equivalent of glucuronic acid was transferred for each equivalent of uridine.

$$\text{UDPguc} + \text{aminophenol} \rightarrow \text{UDP} + \text{aminophenol-glucuronide} \qquad (23)$$

Concentrates of UDP-glucuronic acid were obtained by precipitation of the barium salt with ethanol and purification by paper chromatography. Adenylic acid can be removed with a cation exchange resin (33). Purification with anion exchange resins was found to lead to great losses of the active substance.

Smith and Mills (33) investigated the action of pyrophosphorylases on UDP-glucuronic acid. Neither the yeast enzyme or that from liver nuclei had any action and the results were also negative starting with UTP and α or β glucuronic acid-1-phosphate. The absence of a pyrophosphorylase indicated that there should exist some other route leading to the synthesis of UDPguc, for instance the oxidation of UDPG. Such an oxidation was indeed discovered by Strominger, Kalckar, Axelrod and Maxwell (77). On incubation of a liver extract with UDPG and DPN a reduction of the latter took place and the product acted as glucuronide donor when mixed with another liver fraction and a suitable acceptor such as aminophenol or morphine. The microsome fraction of liver was used for the transferring enzyme and the UDPG oxidizing enzyme was extracted from liver acetone powder and purified 150 fold. No evidence for the accumulation of an intermediate was obtained. Such an intermediate would be expected since the oxidation of the terminal $CH_2OH$ group of glucose to COOH, should be a two step process. This intermediate would be the type of substance which is required for passing from the D to the L series of sugars as has been postulated for the synthesis of ascorbic acid (78), but it would be idle to speculate further at this point.

An interesting point about reaction (*23*) is that in phenol glucuronides the glycosidic linkage is usually β and if it is α in UDPguc, an inversion takes place in contrast to what occurs in the formation of sucrose where an α glucosidic bond is formed.

It is likely that UDPguc has an α linkage because it arises by oxidation of UDPG where the glucose is α. If such is the case UDPguc would be expected to give a cyclic glucuronic acid phosphate ester. Storey and Dutton (79) have investigated this point and although they found that UDPguc, like UDPG, becomes inactivated on chromatography with the ethanol-ammonia they were unable to detect the degradation products. However they report that the lack of material did not allow the performance of the definitive experiments.

The microsome fraction of liver has been found to catalyse the transfer of glucuronic acid from UDPguc to steroids, thyroxine and phenolphtaleine (80).

### *Uridine diphosphate hexosamine compounds*

*UDP-acetylglucosamine.* — UDPag was first isolated from yeast by Cabib *et al.* (81) and has since been found in animal tissues (41, 33) and in tumors (5). Its chemical properties are very similar to those of UDPG except that it is more stable to alkali. By heating at 100° C. in N barium hydroxide, it is decomposed into UMP5′ and acetylglucosamine-1-phosphate. It can be separated from UDPG by chromatography on paper or with anion exchange resins. As to the metabolic role of UDPag it has already been mentioned that it can be synthetized from UTP and acetylglucosamine-1-phosphate by a specific pyrophosphorylase. Another enzyme described below can transform it into UDP-acetylgalactosamine. Moreover Glaser and Brown (82) have presented evidence indicating that together with UDP-glucuronic acid it is involved in the biosynthesis of hyaluronic acid of the Rous sarcoma.

*UDP-acetylgalactosamine.* — Besides UDPag liver contains UDPaga (83, 84) that is an isomer differing in the configuration of carbon atom 4 of the acetylhexosamine residue. The chemical properties of the two compounds are so similar that it has been impossible to separate them. Nevertheless after mild acid hydrolysis the sugar moiety could be studied by suitable methods and was found to consist in a mixture of about 75 % acetylglucosamine and 25 % acetylgalactosamine. The latter was identified by paper chromatography on borate treated paper, by chromatography after deacetylation, and by chromatography of the pentose obtained by degradation with ninhydrin.

The presence of the two isomers UDPag and UDPaga in liver suggested that there might be an enzyme catalys-

ing their interconversion. This has proved to be the case since on incubation of UDPaga obtained from yeast (free from UDPaga) with a crude liver extract part of the acetylglucosamine residue becomes transformed into acetylgalactosamine.

*Hexosamine metabolism.* — The finding of a liver enzyme catalysing the interconversion of UDPag into UDPaga enhances the similarity of the enzymic reactions which take place in the glucose and in the acetylglucosamine series (see Dorfman (85) for a review on hexosamine metabolism). A scheme for the transformations of hexosamines would be as follows :

$$\text{Hexose-6-phosphate} \xrightarrow[(a)]{} \text{glucosamine-6-phosphate} \xrightarrow[(b)]{} \text{acetylglucosamine-6-phosphate} \xrightarrow[(c)]{} \text{acetylglucosamine-1-phosphate} \xrightarrow[(d)]{} \text{UDPag} \xrightarrow[(e)]{} \text{UDPaga}$$

Reaction (*a*) is the synthesis of glucosamine-6-phosphate from glutamine and hexosephosphate which has been studied with an enzyme obtained from a mold (*Neurospora* sp.) (86). It is also known that glucosamine-6-phosphate can be formed by another mechanism involving ATP and hexokinase (87).

The acetylation of glucosamine-6-phosphate with acetyl coenzyme A has been obtained with crude neurospora extracts (86) and with a specific enzyme obtained from yeast (88).

As to the reversible conversion of acetylglucosamine-1-phosphate to the 6-phosphate (86, 89) it is catalysed by an enzyme system different from phosphoglucomutase which can use glucose-1,6-diphosphate as activator. During the reaction glucose-1,6-diphosphate is converted to acetylglucosamine-1,6-diphosphate. The enzyme which has been studied in detail (89) was obtained from neurospora. Extracts from animal tissues have a weak phosphoacetyl-glucosamine-mutase activity.

Other enzyme(s) present in liver and kidney can convert acetylglucosamine-6-phosphate to fructose-6-phosphate and ammonia. Neither glucosamine-6-phosphate or acetamide are intermediates in the reaction. The same enzyme preparations can transform glucosamine-6-phosphate into fructose-6-phosphate provided a catalytic amounts of acetylglucosamine-6-phosphate are added (Cardini and Leloir, unpublished).

As to reaction (*d*) involving UDPag pyrophosphorylase it has already been mentioned, as has also reaction (*e*) leading to UDP-acetylgalactosamine.

*Other UDP hexosamine compounds.* — Park and Johnson (90) observed that in *Staphylococcus aureus*, penicillin leads to an increase in labile phosphate containing compounds. In the first paper it was reported that the compound absorbed strongly in the ultraviolet with a maximum at 262 mμ and yielded uracil and reducing substance on acid hydrolysis.

These studies were followed by Park (91) who succeeded in isolating three different UDP containing substances. The sugar residue of Park's compound I gives a positive Morgan and Elson acetylglucosamine reaction and appears to have an acid group. The other two substances appear to be identical with compound I but, in addition, contain the following aminoacids : one molecule of L-alanine in compound II and a peptide composed of one L-lysine one D-glutamic acid and three alanine residues in compound III.

A study of the incorporation of uracil-$^{14}$C into those compounds has been reported by Strominger (92). Without penicillin the label was rapidly incorporated into the soluble nucleotides and in the uracil and cytosine of nucleic acid. With penicillin there was more radioactivity in the soluble nucleotides and less in the nucleic acids. That is as if penicillin inhibited the conversion of soluble nucleotides into nucleic acids. Such results were obtained with either *Staph. aureus* or *Lactobacillus helveticus*.

Strominger (93) also reported a study of the hexosamine containing compounds of hen oviduct. Besides UDPag and GDPM two new compounds were detected. In one the sugar residue appeared to be acetylglucosamine-6-phosphate and in the other acetylhexosamine with a sulfate group.

### *Future outlook*

The rate at which results are appearing leads us to expect the clarification of many problems which have interested workers for a long time. Besides the uridine compounds described here we know the mono-, di- and triphosphates of guanosine and cytidine. Guanosine-diphosphate-mannose has been isolated (94) and the function of cytidine diphosphate choline as a donor of phosphorylcholine has been established (95). Problems like the mechanism of the synthesis of lactose seem to be on the verge of clarification.

In the field of polysaccharide synthesis UDPG has an indirect role. It acts as a glucose donor to form sucrose and the latter can be used, by several known enzymes, for the synthesis of glucose or fructose polymers. It is not known whether UDPG can also act as a direct donor to polysaccharides.

Many of the intermediate steps leading to the formation of UDPguc and UDPag are now known. The next step will be to elucidate the mechanism by which the glucuronic acid and acetylglucosamine residues are introduced into hyaluronic acid. Do the UDP-compounds act as direct donor ? or is there an intermediate disaccharide ?

The UDP hexosamine sulfate found in oviduct is of great interest in relation to sulfate containing polysaccharides like chondroitin sulfate. Since it has been found that mucopolysaccharides with different amount of sulfate group can be isolated it has been assumed that the sulfate group is introduced after the polymer is formed (96), but it is also possible that enzymes can introduce the sulfated or non sulfated monomer giving rise to products of variable sulfate content.

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# Uridine diphosphate glucuronic acid

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(*Received* 5 *August* 1955)

First of all, we wish to pay tribute to Dr. Leloir and his collaborators, who, by their outstanding contributions to the subject of this symposium, have opened up new vistas in metabolism and have laid the foundations for so much subsequent work.

The main uncertainty in the structure of UDPglucuronic acid is the configuration of the glucuronic acid 1-phosphate linkage. Although there is not any direct chemical evidence that it is α, two lines of enzymic evidence point to this conclusion. The oxidation of UDPG to UDPglucuronic acid in presence of DPN (1) has already been mentioned. In studies with β-glucuronidase Levvy and Marsh (2) showed that, of the glucuronic acid 1-phosphates, only the β-isomer was hydrolysed by the enzyme. Since UDPglucuronic acid did not inhibit the hydrolysis of phenolphthalein glucuronide by β-glucuronidase (3), it cannot be a substrate for the enzyme and hence would be expected to have the α-configuration.

The possible role of β-glucuronidase in glucuronide synthesis has been debated for many years. Whatever the physiological significance of this enzyme, it now seems quite clear that it does not play any part in the synthetic pathway for glucuronides now revealed. Contrary to a recently expressed opinion (4), there are now few, if any, unexplained discrepancies between the results obtained with liver slices and homogenates.

Although it was previously suggested (5) that the reaction studied was a simple transfer of glucuronic acid with the formation of the glucuronide and UDP, with the facilities then available and accordingly using mainly crude homogenates we were unable to detect the formation of the last named compound. Using microsomal preparations, Isselbacher and Axelrod (6) were able to show that UDP was formed in amounts equimolar with the free steroid disappearing, the UDP being determined enzymically. It would undoubtedly be of great value, especially for studies on the specificity of the acceptor, if further purification of the enzyme system transferring the glucuronic acid could be achieved. Following upon our demonstration (3) that the activity was associated with the particulate components of the cytoplasm, we have made numerous attempts to bring the enzyme into solution; but it appears to be very readily inactivated by many of such treatments and success has not yet been achieved. However, microsomal preparations may be freeze dried without much loss of activity.

In most of the *in vitro* work on glucuronide synthesis the glucuronidogenic agent has been either a phenol (*e.g. o-* or *m*-aminophenol) or a hydro-aromatic alcohol (menthol or borneol). From time to time, objections have been raised as to whether such compounds, especially *o*-aminophenol, are really representative of natural substrates for the glucuronide synthesizing system. Although for the simple glucuronides, which are excretory products, such criticism is of doubtful validity, various other acceptors have indeed been investigated. The results of some preliminary experiments are shown in table I. When androsterone, allopregnanediol and stilboestrol are incubated with UDPglucuronic acid and

TABLE I

*Formation of ethyl acetate-soluble, Tollens-positive conjugates by liver homogenate in presence of UDPglucuronic acid*

| | Glucuronic acid conjugated (μg.) | | |
|---|---|---|---|
| | Androsterone | Stilbœstrol | Allo-pregnanediol |
| + UDP glucuronic acid | 6.0 | 5.1 | 2.0 |
| + UDP glucuronic acid (added after incubation) . . . . . . . | 2.1 | 1.3 | 0.9 |
| + UDP glucuronic acid (substrate added after incubation) . . . . | 0.4 | 0.5 | 0.3 |

a liver homogenate, there is a marked increase, compared with the controls, of material extractable by ethyl acetate and giving a positive naphthoresorcinol reaction. Similar results have also been reported by Isselbacher and Axelrod (6). Furthermore, Dutton (7) has shown that UDPglucuronic acid acts as glucuronic acid donor to carboxylic acids in the formation of ester glucuronides.

The possible role of UDPglucuronic acid and other UDPglycosyl compounds in the biosynthesis of mucopolysaccharides emphasizes the necessity for further studies on the specificity of the glucuronic acid acceptor. We have previously suggested (5) that glucuronide formation might perhaps represent a diversion of 'active' glucuronic acid which would normally be used for mucopolysaccharide formation. A number of compounds have now been tested for their influence upon glucuronide synthesis in presence of UDPglucuronic acid, *o*-aminophenol and liver homogenates. UDPG, glucosamine, N-acetylglucosamine, the α- and β-glucuronic acid 1-phosphates, glucose-1-phosphate and glucosamine-6-phosphate (these last two compounds also after incubation with phosphoglucomutase) were without significant effect even when present in considerable excess over the UDPglucuronic acid. Such studies might be of value if repeated with purified enzyme preparations when these become available. On the other hand, if the diversion theory is incorrect, an entirely different enzyme system might be involved in further transformations of UDPglucuronic acid.

Of the normal animal tissues studied, only the liver has been found to contain either UDPglucuronic acid or the enzyme system, but they may be presumed present also in the kidney in view of the small but definite synthetic activity shown by slices of this organ. The demonstration of UDPglucuronic acid in the Flexner-Jobling carcinoma (8) is therefore of much interest. We have also found small amounts in the RB 3 sarcoma. Preliminary observations made some time ago gave an indication that it was present in certain organisms which form extracellular polysaccharide containing glucuronic acid.

It is interesting to recall that whilst the investigation of certain enzyme systems led to the discovery and isolation of UDPG and UDPglucuronic acid, ion-exchange chromatography has now provided many other compounds of UDP and the problem now is rather to find the enzyme systems in which they are active.

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## Synthesis of disaccharides with pea preparations

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(*Received* 9 *September* 1955)

The discovery of the coenzyme UDPG in yeast by Leloir and coworkers (1) was an important contribution to the progress of biochemistry. As we have learned from his excellent review of the 'uridine coenzymes', UDPG is of widespread occurrence in living cells, and plays a vital role in a number of hitherto unexplained biochemical reactions, namely, the glucose-galactose transformation, sucrose synthesis, the synthesis of trehalose and other oligosaccharides.

About a decade ago Dr. Doudoroff and his coworkers (2) in our laboratories in Berkeley, obtained an enzyme preparation from *Pseudomonas saccharophila* capable of catalyzing the formation of sucrose from α-D-glucose-1-phosphate and D-fructose. However, it was not possible to detect such a reaction in plants. Experiments with Canna leaves by Putman and Hassid (3), using $^{14}$C-labeled hexose sugars, indicated that phosphorylated derivatives of both D-glucose and D-fructose serve as precursors in the synthesis of sucrose, and that free D-fructose is not involved in the reaction. Buchanan *et al.* (4, 5) first presented evidence indicating the presence of sucrose phosphate in plants, and suggested that the

phosphorylated sucrose is formed as a result of the reaction between UDPG and fructose phosphate. Their data indicated that the phosphorylated fructose resulting from the hydrolysis of sucrose phosphate was probably D-fructose-1-phosphate.

Leloir and Cardini (5, 6) showed that enzyme preparations from wheat, corn, peas and bean germs catalyze the reversible formation of sucrose from UDPG and free fructose :

(*1*) UDPG + D-fructose $\rightleftarrows$ UDP + sucrose.

They also demonstrated (7) that some pea preparations contain an enzyme which will form sucrose phosphate when fructose-6-phosphate is substituted for D-fructose :

(*2*) UDPG + D-fructose-6-phosphate $\rightleftarrows$ UDP + sucrose phosphate.

The enzymes causing reactions (*1*) and (*2*) could not be completely separated, but sufficient evidence was presented to conclude that two enzymes are involved.

In the present communication, using preparations from green peas, we confirmed the synthesis of sucrose from UDPG and D-fructose, and of sucrose phosphate from UDPG and D-fructose-6-phosphate. We have also shown that these preparations produce two other disaccharides, D-glucosyl-D-xyluloside and D-glucosyl-D-rhamnuloside (*), according to the reactions :

(*3*) UDPG + D-xylulose $\rightleftarrows$ UDP + D-glucosyl-D-xyluloside, and

(*4*) UDPG + D-rhamnulose $\rightleftarrows$ UDP + D-glucosyl-D-rhamnuloside. The disaccharide in reaction (*c*) is probably identical with the one formed by the action of an enzyme present in *Pseudomonas saccharophila* from D-glucose-1-phosphate and D-xylulose (8).

The enzyme preparations were made as follows : 500 g. of fresh peas were placed in 150 ml. of water, homogenized in a Waring blendor, the homogenate was centrifuged and the supernatant solution was fractionated with ammonium sulfate. The precipitate occurring between 20 and 50 % ammonium sulfate saturation was dissolved in a minimum of water, dialyzed for 2 hours against distilled water and then overnight against 0.05 M phosphate buffer, pH 7. The solution was adjusted to pH 5, the resulting precipitate dissolved in water, dialyzed again against 0.05 M sodium versenate (ethylene diamine tetraacetate), pH 7, overnight, and then against phosphate buffer at the same pH to remove the versene. The final solution, which had a volume of 7 ml., contained 5 mg. N /ml.

The products of the reactions were determined by analytical methods similar to those used by Leloir and Cardini (1). We found that with green pea preparations sucrose formation by reaction (*1*) was stimulated by $10^{-3}$ M $Mg^{++}$. Sucrose phosphate synthesis by reaction (*2*) did not occur unless $Mg^{++}$ was added.

For the preparation of the D-glucosyl-D-xyluloside a mixture consisting of 0.9 μ-moles of UDPG, 8 μ-moles of D-xylulose, and 0.4 ml. of enzyme preparation from peas was incubated for 6 hours. The enzyme was inactivated by adding 5 volumes of ethanol and heating the digest. The mixture was centrifuged, the solution evaporated, and the resulting sirup chromatographed two-dimensionally on paper, using butanol : acetic acid : water and phenol : saturated water as developing solvents. After spraying with *p*-anisidine hydrochloride, a red spot appeared on the chromatogram which coincided with that of D-glucosyl-D-xyluloside prepared by the action of sucrose phosphorylase from *P. saccharophila* on D-glucose-1-phosphate and D-xylulose. A control without UDPG produced only a D-xylulose spot. Elution of the disaccharide spot and hydrolysis with 0.1 M HCl at 100° C. for 10 minutes and chromatography of the products yielded spots which were identified by their $R_f$ values as D-glucose and D-xylulose.

The rate of D-glucosyl-D-xyluloside formation relative to that of sucrose synthesis was estimated by determining its synthesis at several time intervals under similar conditions. Samples consisting of 2.25 μ-moles of UDPG, 40 μ-moles of D-xylulose and 0.25 ml. of enzyme solution in a total volume of 0.45 ml. were incubated at 37° C., and the D-glucosyl-D-xyluloside produced was determined colorimetrically at various intervals by a modification of the Dische and Borenfreund (9) reaction. Analysis of the samples showed the formation of 0.60, 0.95, and 1.2 μ-moles disaccharide after 30, 60, and 120 minutes, respectively. Under similar conditions D-glucosyl-D-xyluloside is synthesized at a considerably slower rate than sucrose. Using the same UDPG concentration and a D-xylulose concentration 16 times greater than that of D-fructose in the sucrose reaction, the rate of formation of D-glucosyl-D-xyluloside was still slower than that of sucrose.

When a mixture of 2.25 μ-moles of UDPG, 30 μ-moles of D-rhamnulose and 0.25 ml. of enzyme was incubated in a total volume of 0.45 ml. of water under the same conditions as the mixture containing the D-xylulose, 0.70 μ-moles of D-glucosyl-D-rhamnuloside was obtained after 30 minutes and 0.91 μ-moles after 60 minutes. The rate of formation of this disaccharide was similar to that of the D-glucosyl-D-xyluloside.

Thus, it appears that peas contain an enzyme (probably the same as the one responsible for the reaction of UDPG with D-fructose to form sucrose) which is capable of producing D-glucosyl-D-xyluloside from UDPG and xylulose, and D-glucosyl-D-rhamnuloside from the same nucleotide and D-rhamnulose.

When sucrose was first enzymatically synthesized from glucose-1-phosphate and fructose by the *P. saccharophila* enzyme, we were inclined to believe that this mechanism of sucrose formation was probably a universal process. However, since the equilibrium of this reaction favors α-D-glucose-1-phosphate formation, such a mechanism could not explain the accumulation of high concentrations of sugar that occur in many plants. Besides, this enzyme could not be detected in higher plants. The newly discovered mechanisms for sucrose and sucrose phosphate synthesis by Leloir and Cardini, the first involving UDPG and D-fructose and the second, the same nucleotide and fructose-6-phosphate, both have a more favorable equilibrium for sucrose accumulation. The second reaction,

(*) The D-rhamnulose was obtained by Drs. N. Palleroni and M. Doudoroff through isomerization of D-rhamnose with a bacterial enzyme (mannose isomerase) preparation from *P. saccharophila*.

resulting in the formation of sucrose phosphate, which may lead to the formation of sucrose through hydrolysis of sucrose phosphate by phosphatase present in the plants would be especially favorable for sucrose accumulation, because this hydrolytic step is practically irreversible. It is likely that in plants such as sugar beets where large concentrations of sucrose accumulate, an intermediate sucrose phosphate is synthesized in the leaves, which is immediately hydrolyzed by phosphatase to free sucrose and the latter is than translocated to the root.

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# Uridine nucleotides in animals and bacteria


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(*Received* 9 *September* 1955)

In 1949 and 1950 Caputto, Leloir, Cardini and Paladini (1) studying galactose metabolism in yeast, and Park and Johnson (2) studying the effects of penicillin on bacteria, independently observed the occurrence of acid-soluble uracil-containing compounds in living tissues. These were the first observations of the occurrence (other than in nucleic acid) of pyrimidine nucleotides. That the observations should have come from such different approaches was perhaps an augury of the variety of phenomena which are now known to involve uridine nucleotides. Their observations were the immediate impetus to the investigations which will be summarized here, and it is therefore a particular pleasure to present this supplement following Dr. Leloir's report.

At about this same time the application of anion exchange chromatography to the separation of nucleotides (3), and a simple method for the detection of ultraviolet absorbing substances on paper chromatograms (4-6) were described. These technical advances have contributed very greatly to further progress in the field.

## *Microbial uridine pyrophosphate N-acetylamino sugar compounds*

The three uridine nucleotides which Park isolated from penicillin-inhibited *Staphylococcus aureus* (7) and later found in normal *S. aureus* in smaller amounts (8) are unique biological substances in that two of them contain a portion of a polynucleotide linked through covalent bonds to amino acids. An improved method of preparing these compounds (figure 1) and a convenient quantitative procedure for measuring their accumulation in bacterial extracts have been developed. Employing these methods evidence which suggests that accumulation of the nucleotides may be closely related to a point of inhibition of penicillin within the bacterial cell has been obtained (9). Uridine pyrophosphate N-acetylamino sugar compounds

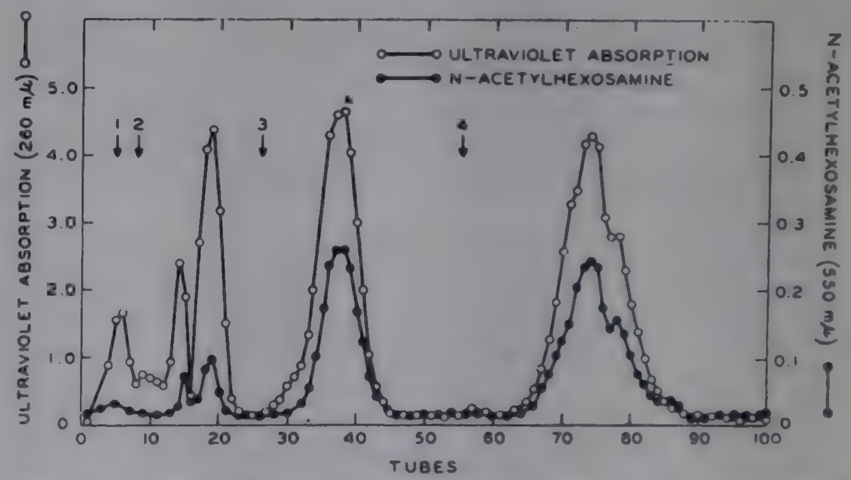


FIG. 1. — Preparation of nucleotides from penicillin-treated *S. aureus* by anion exchange chromatography of extract (Dowex-1 chloride, 2 % cross-linked). The nucleotides with a peak at tube 38 is Compound 3; that at tube 72 is a mixture of Compounds 1 and 2. These preparations contain 5-15 % ultraviolet absorbing impurities.

accumulate in penicillin-inhibited *Lactobacillus helveticus 335* also (9) (table I) but amounts of these compounds sufficient for detailed structural studies have not yet been available. As to their function, the experiments with uracil-2-$^{14}$C (9, 10) which Dr. Leloir has mentioned strongly suggest that the accumulation of the compounds in both *S. aureus* and *L. helveticus* is a consequence of an inhibition by penicillin of nucleic acid synthesis or

(*) Supported by the Markle Foundation and the Commonwealth Fund.

TABLE I

*Accumulation of N-acetylamino sugar-containing nucleotides in penicillin-treated* L. helveticus 335

| Penicillin (µg./ml.) | Hours | µg-moles/l. culture |
|---|---|---|
| 0 | — | 3.6 |
| 1.3 | 2 | 8.6 |
| 2.5 | 2 | 12.2 |
| 2.5 | 4 | 15,3 |

turnover. Of course, the interpretation that the compounds are precursors of at least a portion of the bacterial nucleic acid is an attractive one, but the experiments clearly do not differentiate the possibility that the accumulating compounds are in equilibrium with a nucleic acid precursor, *i.e.* that their accumulation is the result of the diversion into a side path of metabolites which normally find their way into nucleic acid. In any case, an hypothesis is that the compounds are intermediates common to the synthesis of a part of the bacterial nucleic acid and protein.

Several structural features of the *S. aureus* compounds remain to be elucidated, viz. : the nature of the acetylamino sugar, believed by Park to be an acetylamino uronic acid, and a more exact structure for the peptide in Compound 3 which contains 3 residues of DL-alanine, 1 of D-glutamic acid and 1 of L-lysine. This peptide is linked to the acetylamino sugar through its amino end. Recently (*), it has been found that in N HCl at 38° C., Compound 3 undergoes a limited hydrolysis (11). One of the fragments which contains all the amino acids but no amino- or acetylamino-sugar has alanine as the N-terminal amino acid (determined by a modification of the Edman phenyl*iso*thiocyanate procedure, 12). Alanine is, therefore, the amino acid which is bonded to the acetylamino sugar in Compound 3. Since Compound 2 contains only alanine, this finding is compatible with the hypothesis that Compound 2 is a biological precursor of Compound 3.

Alanylalanine has also been identified as a component of the peptide, and other evidence suggests that alanine is also the C-terminal amino acid. These findings limit the number of possible sequences for the peptide to four (twenty could be constructed initially), viz. : ala-glu-lys-ala-ala, ala-lys-glu-ala-ala, ala-ala-glu-lys-ala and ala-ala-lys-glu-ala. No information has been obtained yet about the optical rotation of the various alanines. It is apparent from the fact that there are 3 residues of DL-alanine that more than one species of peptide exists. This study has proceeded on the assumption that the species have the same sequence and differ only in the optical rotation of the alanines at one or more positions.

*Uridine nucleotides from hen oviduct*

A systematic investigation of the uridine pyrophosphate N-acetylamino sugar compounds of several animal tissues was undertaken with a view to the possibility of the occurrence in them of compounds of the type which Park had found in *S. aureus*. Although compounds of this type were not detected in hen oviduct or in rabbit liver, a number of other interesting nucleotides were encountered in oviduct (13).

Anion exchange chromatography was employed for the systematic investigation of acid-soluble nucleotides. The eluent from the column was examined both for N-acetylamino sugar (14) and for nucleotide (260 mµ absorption). There, and sometimes four, peaks in the elution diagram of oviduct extract (figure 2) contained

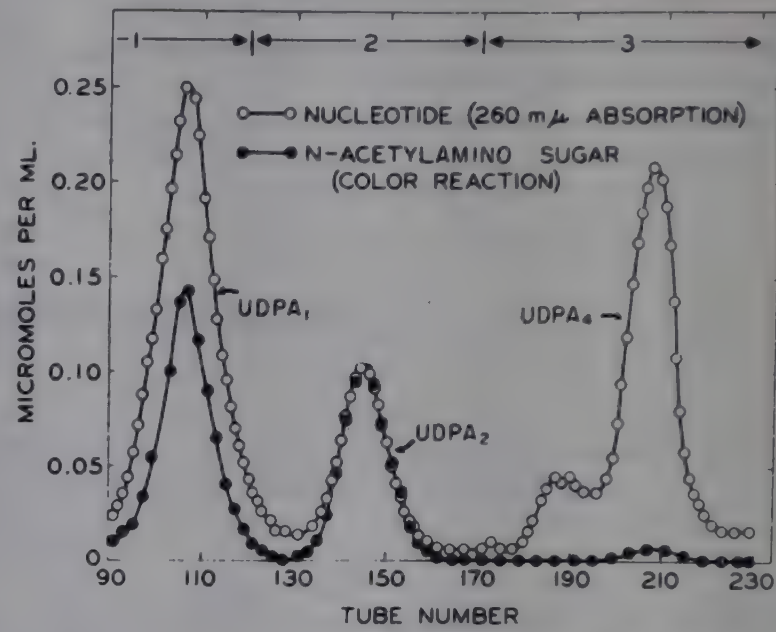


FIG. 2. — Anion exchange chromatogram of hen oviduct extract.

both N-acetylamino sugar and ultraviolet absorption. The first peak was found to contain uridine diphosphate acetylglucosamine, originally identified in yeast by Cabib, Leloir and Cardini (15) and also found in animal tissues by several other authors. This peak also contained two other nucleotides, guanosine diphosphate mannose (16, 17) and uridine diphosphate acetylgalactosamine (13, 18).

The compound in the second peak was found analytically to contain uridine diphosphate acetylglucosamine and an additional residue of phosphate, stable to hydrolysis in N HCl. Hydrolysis studies showed that the additional phosphate residue was attached to the acetylglucosamine, and a fragment was obtained from the nucleotide which contained acetylglucosamine and two residues of phosphate, one labile and one stable to hydrolysis in N HCl. This compound was sent to Buenos Aires where Dr. Reissig found that it would activate *Neurospora* sp. phosphoacetylglucosamine mutase. Since then Dr. Reissig has prepared acetylglucosamine-1, 6- diphosphate by another method, and has shown that both compounds activate the mutase reaction in similar fashion. Neither compound, incidentally, activates phosphoglucomutase. The oviduct nucleotide is, therefore, presumably uridine diphosphate (1)-acetylglucosamine-6-phosphate (UDPAGP).

The compound in the fourth peak contains uridine diphosphate acetylgalactosamine and one residue of sulfate, which is attached to the amino sugar (UDPAGalS). Nothing further is known about the position of

(*) These experiments were carried out at the Carlsberg Laboratory, Copenhagen, and I am much indebted to Professor K. Linderstrom-Lang for his hospitality.

the sulfate, but a more detailed investigation of the structure of the amino sugars of UDPAGP and UDPAGalS is now being carried out in collaboration with Dr. R. W. Jeanloz.

The structure of the latter compound points to a possible function, that the compound may be a precursor of chondroitin sulfate. As Dr. Leloir has already mentioned, schemes for the synthesis of chondroitins with very varied sulfate contents can be envisioned when the possibility of the participation of both UDPAGalS and UDPAGal in synthetic reactions is considered.

As for UDPAGP, if the mucopolysaccharides do not contain 1 : 6 branches (19), its possible function is obscure. However, it seems possible that a hyaluronic acid phosphate might be a precursor of hyaluronic acid.

Mucopolysaccharides have recently been found in oviduct (20). Oviduct is also very rich in glycoproteins which coincidentally contain chiefly acetylglucosamine and mannose in the polysaccharide (21). Perhaps the nucleotides are involved in their synthesis. However, the presence of protein in these molecules and the fact that so little is known about the linkage between protein and polysaccharide introduces another order of complexity into the synthetic problem.

### *Enzymatic synthesis of uridine diphosphate glucuronic acid (UDPGA)* (*)

Following the demonstration by Dutton and Storey of the occurrence of uridine diphosphate glucuronic acid (UDPGA) and its function as a glucuronide donor (22), Smith and Mills were unable to demonstrate a synthesis of this compound by a pyrophosphorylase mechanism (23). This suggested that the biosynthetic route to UDPGA might be *via* a direct oxidation of uridine diphosphate glucose (UDPG). It was possible to demonstrate that a fraction from liver homogenate obtained by relatively brief centrifugation (containing both microsomes and supernatant) would catalyze the synthesis of *o*-aminophenol glucuronide when supplemented with UDPG, $DPN^+$ and *o*-aminophenol (24). Experiments with morphine as acceptor, similarly suggested the synthesis of morphine glucuronide. With further fractionation of this crude system, it was evident that the enzyme catalyzing the oxidation of UDPG was in the supernatant while the catalyst for the transfer of glucuronate from UDPGA to the acceptor was in the microsomes. The observations are summarized in figure 3.

Using as an assay the reduction of $DPN^+$ by UDPG, the oxidizing enzyme was purified 200-400 fold, and evidence that the product of the oxidation is UDPGA has been obtained. It may be pointed out that the oxidation of UDPG to UDPGA involves the reduction of two moles of $DPN^+$ or the transfer of 4 electrons, and might be expected to proceed in two separable steps. However, even the best preparation, which was 420-fold purified, catalyzed a smooth reduction of two moles of $DPN^+$ with no evidence of discontinuity to suggest the participation of more than one enzyme. Attempts to trap a possible aldehyde intermediate with semicarbazide or hydrazine were unsuccessful. A similar case, the oxidation of the amino alcohol, histidinol, to the amino acid, histidine, has recently been extensively studied by Adams (25). Adams was able to synthesize the intermediate aldehyde, histidinal. From studies of the oxidation and reduction of this compound catalyzed by a purified bacterial enzyme, from inhibitor studies and from studies of bacterial mutants blocked at this metabolic step he has found no evidence for the participation of more than one enzyme in the two-step oxidation.

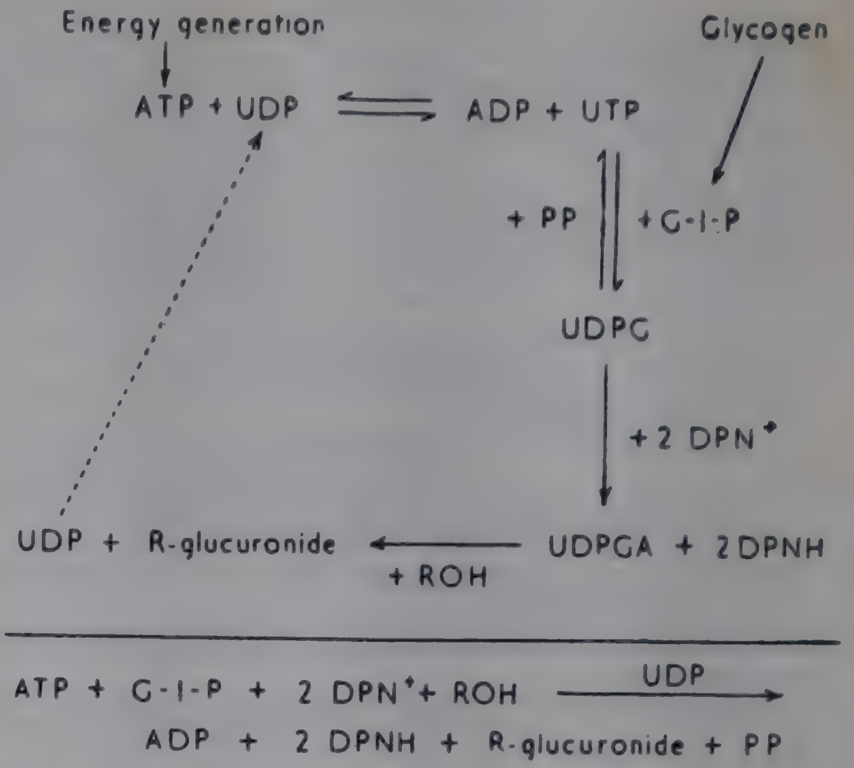


FIG. 3. — The overall reaction in glucuronide synthesis : a UDP cycle. This schematic representation serves to emphasize that the source of the glucuronide is glucose-1-phosphate, that the energy for the synthesis can be derived from ATP, and that a uridine nucleotide can operate catalytically in the overall reaction. A similar cyclic mechanism can operate in the synthesis of sucrose or of trehalose phosphate.

The synthesis of UDPG (26, 27) and its oxidation to UDPGA is the beginning of a third metabolic pathway for glucose in animal tissues. In addition to glucuronide synthesis, there are other interesting directions in which UDPGA or some relative might lead, particularly the biosyntheses of polysaccharides and of ascorbic acid.

The purified UDPG dehydrogenase should also have a number of interesting practical applications. As Dr. Leloir has suggested, further study of the galactowaldenase reaction has been impeded by the lack of a separate and convenient assay for this reaction. It may be suggested that the transformation of UDPGal to UDPG could be conveniently followed by coupling the reaction to UDPG dehydrogenase (since UDPGal is oxidized very slowly, if at all, by this enzyme). As for the preparation of UDPGal, which has been found naturally only in a mixture with UDPG which has not been resolved, it seems likely that this compound can be prepared by oxidizing the UDPG with the aid of the dehydrogenase. The compounds in the resulting mixture should be easily separable.

(*) These studies were carried out in collaboration with Drs. H. M. Kalckar, J. Axelrod and E. S. Maxwell.

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# Galacto-waldenase and the enzimic incorporation of galactose-1-phosphate in mammalian tissues


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(*Received* 20 *September* 1955)

Extracts of galactose-adapted *Saccharomyces fragilis* catalyze the incorporation of galactose-1-phosphate (gal-1-P) into uridylic-bound galactose according to the following equation :

$$(a)\ \text{gal-1-P} + \text{UDPG} \rightleftarrows \text{UDPgal} + \text{G-1-P} \quad \text{(ref. 1, 2)}$$

In the conversion of gal-1-P to glucose-1-phosphate (G-1-P), uridine diphosphate glucose (UDPG) acts catalytically since it can be regenerated by Leloir's galacto-waldenase (3) :

$$(b)\ \text{UDPgal} \rightleftarrows \text{UDPG}$$

Reaction (*a*) is a non-pyrophosphorolytic type of uridyl transfer catalyzed by an enzyme which we call GP-uridyl transferase in contrast to PP uridyl transferase (4) which catalyzes the reversible pyrophosphorolysis of UDPG :

$$(c)\ \text{UDPG} + \text{PP} \rightleftarrows \text{UTP} + \text{G-1-P}$$

Recently we have found large amounts of both GP-uridyl transferase and galacto-waldenase in rat and calf liver. Moreover, since the transferase is able to resist heating to 50° C. at pH 5.9, it can be separated from the heat labile galacto-waldenase. The separation of the two enzymes has made it possible to prepare UDPgal of sufficient purity for use in a simple spectrophotometric assay for galacto-waldenase.

Pure UDPGalactose was isolated by enzymatic means. UDPgal was assayed by conversion to UDPG which was determined quantitatively by oxidation to uridine diphosphate glucuronic acid (5).

Galacto-waldenase has been partially purified from a water extract of calf liver acetone powder by repeated ammonium sulfate fractionation at different hydrogen ion concentrations. It was found that mammary gland from lactating rats contained about 3 times as much enzyme as the corresponding amount of protein from mammary gland of non-lactating rats (see table I). Interestingly enough the same preparations were unable to incorporate gal-1-P into uridyl nucleotide according to reaction (*a*), although they contained PP uridyl transferase and were able to form UDPG and UDPgal from G-1-P and UTP. Similar results were encountered in experiments using brain extracts from two weeks old rats, *i.e.*, active galacto-waldenase and active PP uridyl transferase were present but no reaction occurred between galactose-1-phosphate and UDPG. The liver of these animals contained, in addition to galacto-waldenase and PP uridyl transferase, the enzyme GP uridyl transferase which catalyzes the reaction between gal-1-P and UDPG.

(*) Read by J. L. Strominger.

TABLE I

*Galacto-waldenase in mammary gland*

Assay mixture contained : crude protein fractions from mammary glands 2 mg. protein, UDPgal 0.04 μ-moles, DPN 0.5 μ-moles, cysteine 5 μ-moles, and UDPG dehydrogenase in 0.5 ml. 0.1 N glycine buffer pH 8.6.

| Time (min.) | UDPG formed (μ-moles) | |
|---|---|---|
| | non-lactating | lactating |
| 10 | 0.005 | 0.016 |
| 30 | 0.015 | 0.030 |
| 60 | 0.030 | 0.040 |

It may be worth emphasizing that the waldenase serves not only as a link in the conversion of galactose to general 'fuel' for the cell, but also in converting glucose compound into specific galactosyl compounds such as galacto-lipids, which are the building blocks of structural components of the cell. Some of these problems are under investigation. With the availability of UDP-glycosyl compounds labeled in the glycosyl moiety, which are being prepared by enzymatic synthesis, a new tool has been added to this research.

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# The hexosemonophosphate oxidative pathway of yeast and animal tissues

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(*Received* 16 *April* 1955)

During the past few years there have been remarkable developments in our knowledge of mechanisms for the oxidative metabolism of hexose sugars, which have also led to a great increase in our understanding of pentose metabolism, and have in addition brought those chemical rarities, the heptoses and tetroses, into place in the metabolic picture. The purpose of this introductory paper is to review this recent work in broad outline and to try to make a very provisional estimate of its possible significance for life-processes, particularly in animal tissues. The enzymes of the pathways which will be mentioned here have already been found to be active in a wide range of micro-organisms and higher plants, as well as in animals, so that one can now hardly have much doubt about their ultimate functional importance.

At this point someone is almost certain to be recalling the fate of the enzyme glyoxalase, so widely present in cells and tissues and, after holding a front-line position for a decade, now relegated to obscurity. On the other hand, while the presence of a single enzyme may thus prove to be fortuitous, it is much more difficult to believe that mere chance could account for the widespread presence of a complicated enzyme-chain of reactions, involving many separate steps, which are regularly repeated as a pattern common to such different forms of life as the liver of the rat and the leaves of the spinach. I think it probable that this important point will emerge very clearly.

Although progress has been rapid, there are at present still many gaps, but these are being closed by highly active work in a number of laboratories, many of which are represented at this meeting and whose views we hope to learn.

Here I wish to thank my colleagues Drs. G. E. Glock, P. McLean and G. F. Humphrey for kindly allowing me to make use of their work, now in course of publication.

### *Alternative pathways of carbohydrate metabolism*

It is not intended to spend much time on the historical aspects of the subject which have been fully reviewed elsewhere (1-5). It is here only necessary to note the establishment of the Embden-Meyerhof pathway of glycolysis, as a fundamental pattern of anaerobic breakdown of carbohydrate to lactic acid in animal tissues, and to alcohol, glycerol and other products in microorganisms and plant tissues (6). This system usually requires diphosphoryridine nucleotide (DPN), although some plant tissues, *e.g.*, pea seedlings, have a glyceraldehyde phosphate dehydrogenase which is apparently linked not only to DPN but also to TPN (7, 8). While other variations can occur, the general route is well known. This pathway is inhibited by iodoacetate and fluoride, by selective poisoning of glyceraldehyde phosphate dehydrogenase and enolase respectively. Although pitfalls exist in the use of these inhibitors (6) they are nevertheless a useful guide to the existence of the glycolytic pathway. From this pathway the hexosemonophosphate (HMP) oxidative route diverges at the level of glucose-6-phosphate (figure 1).

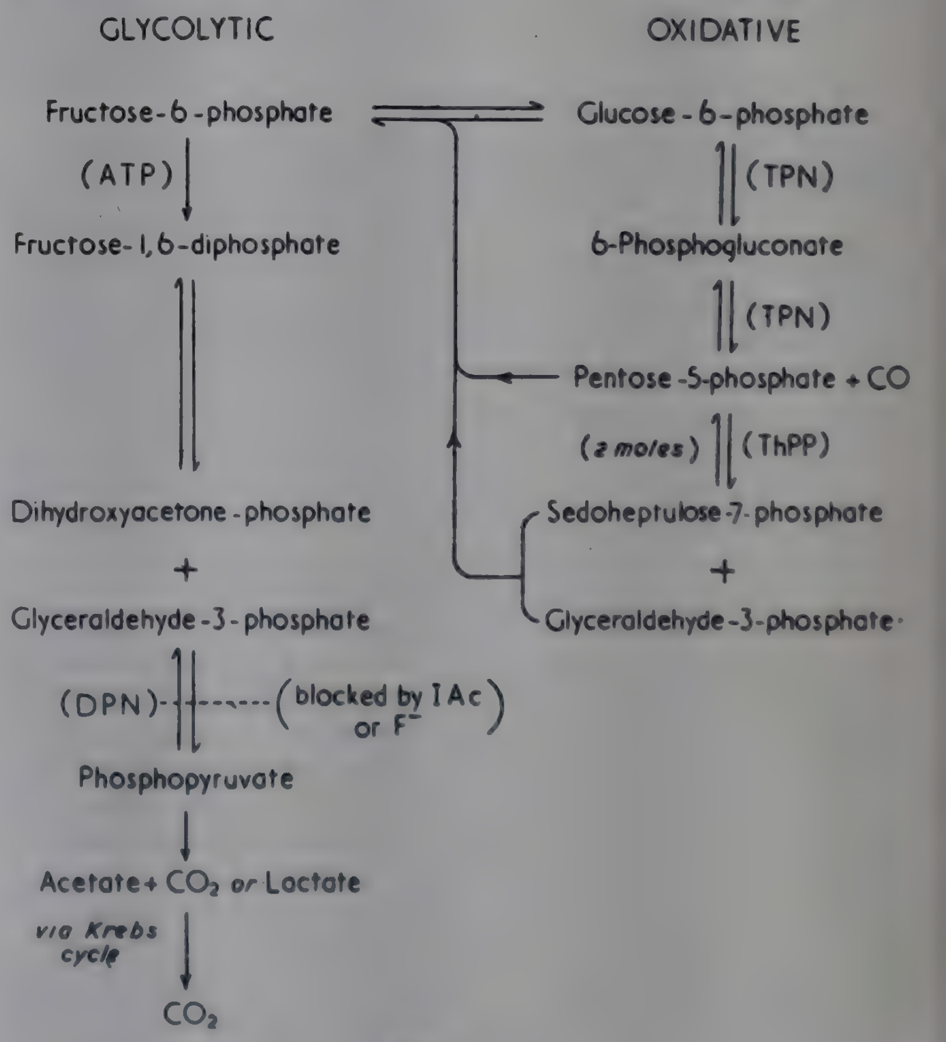


FIG. 1.

Lipmann (9) was the first to observe that the respiration of yeast extract was insensitive to bromoacetate, although the fermentation of such extracts was blocked by this reagent; and somewhat similar resistant respiration has been described (10) in slices of brain and muscle in presence of fluoride or iodoacetate. Shortly before this, Warburg's classical studies on hexose monophosphate oxidation in red-cells and in yeast (11) had led to his discovery of TPN and of the oxidation of glucose-6-phosphate to 6-phosphogluconic acid by a TPN-coupled dehydrogenase, (*Zwischenferment*) of yeast. The reaction, which could proceed further (11), proved to be an oxidative decarboxylation (12, 13) and further study reactions all specifically required TPN, whereas the alcoholic fermentation of D-ribose-5-phosphate required DPN. Other pentose phosphates (D-xylose-5-phosphate (14), D-arabinose-5-phosphate (14), and D-ribose-3-phosphate (18)), were not attacked, or only very slowly in comparison with ribose-5-phosphate. It was therefore suggested in 1938 that D-ribose-5-phosphate was probably the pentose ester formed by oxidative decarboxylation of 6-phosphogluconate in this system (13, 16), rather than the theoretically expected D-arabinose-5-phosphate as suggested earlier by Lipmann (9) on the basis of the evolution of $CO_2$ in the presence of bromoacetate.

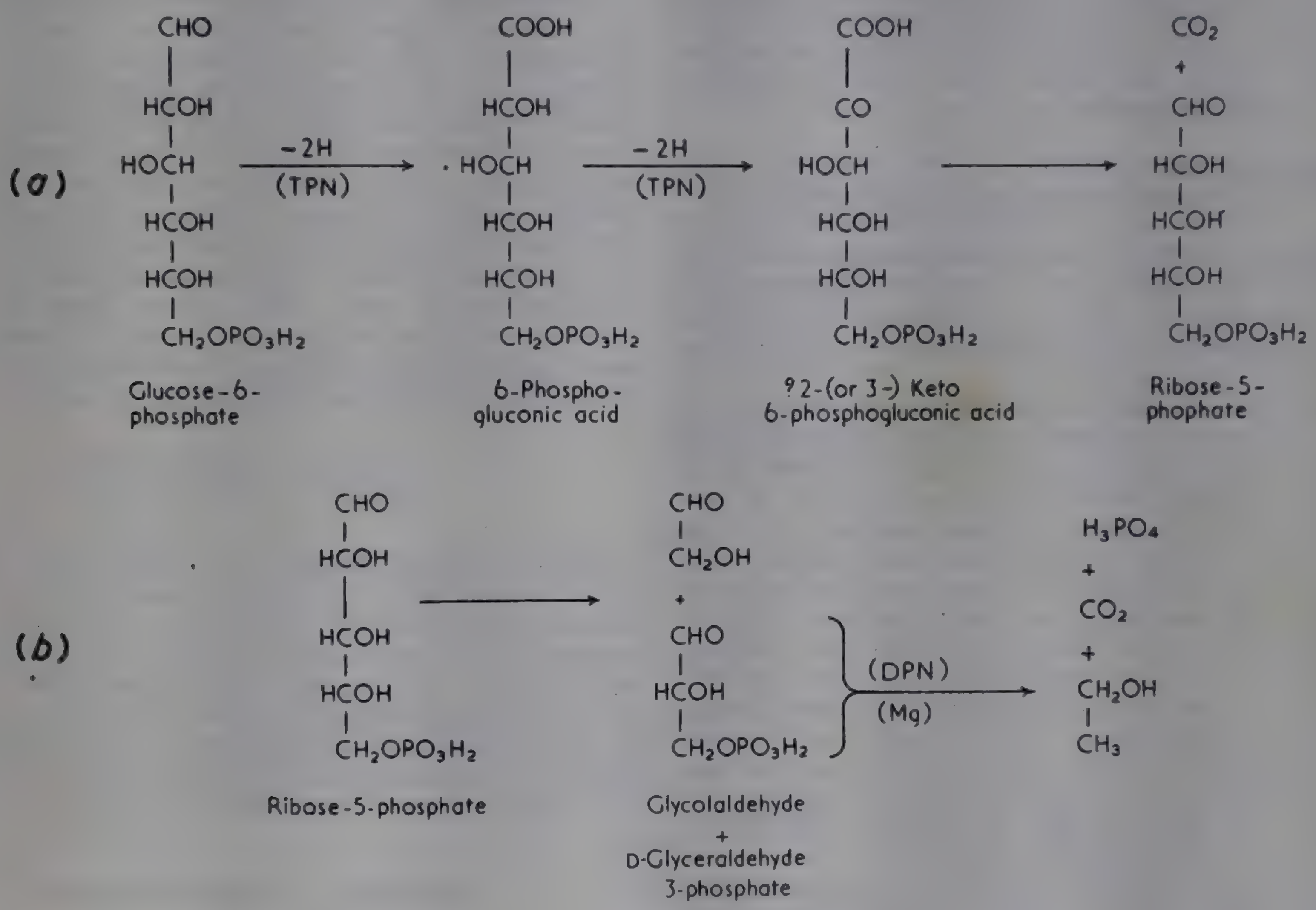


FIG. 2.

provided evidence that a pentose phosphate (see figure 2*a*) resulted (14). In the acide precipitated oxidative enzyme preparation as used by Dickens in 1938 and further studied by Scott and Cohen (15), we now find (16) that phosphopentose breakdown is quite slow, and this probably accounted for the accumulation of pentose then observed. On the other hand the dialysed yeast extract used in the early studies of pentose phosphate fermentation (17) was shown to be highly active in systems which degrade ribose-5-phosphate, and with DPN and $Mg^{++}$ added, fermented it to equimolar amounts of alcohol, $CO_2$ and phosphate (fig. 2*b*). Similarly, the acid precipitated oxidizing enzyme system of yeast was found capable of oxidizing D-ribose-5-phosphate, as well as glucose-6-phosphate and 6-phosphogluconate, yielding carbon dioxide. These three oxidative

As will be seen presently, these apparently simple reactions are in fact much more complex than could have been forseen at the time, but their overall nature is shown in figure 2.

At about this time, Dische (18, 18*a*) had independently shown that haemolysed red blood cells, in the presence of sodium fluoride and bromoacetate, first esterified adenosine and then converted the ribose-phosphate moiety into hexose and triose phosphates, together with an unidentified reducing carbonyl compound. This reaction was later clarified, by isolation of the Robison equilibrium ester formed, by Waldvogel and Schlenk (20). These were the first indications of a fermentative route of pentose phosphate metabolism leading back to the formation of hexose. For further historical details, the reviews already quoted (1-5) should be consulted.

*The oxidation of 6-phosphogluconate*

No further work was done on the mechanism of the oxidative system for 10 years, although Engelhardt and Sakov (21) developed a theory of the Pasteur effect, based on inhibition of the glycolytic pathway, by oxidative suppression of phosphofructokinase activity. Then, starting in 1950, Cohen and Scott (15, 18, 22, 23) and, independently, Horecker and Smyrniotis (24-27), have brilliantly clarified the mechanism by separation and identification of the pentoses formed. In addition to the D-ribose-5-phosphate (15), an alkali-labile ketopentose ester, ribulose-5-phosphate, was found by Horecker and his colleagues (25, 26) to constitute most of the product and to be that first formed by oxidation of phosphogluconate by purified dehydrogenase preparations from yeast and liver. The reaction proceeds according to the reversible equation :

$$\text{6-phosphogluconate} + \text{TPN} \rightleftarrows \text{pentose phosphate} + CO_2 + \text{TPNH}_2.$$

This reaction could be made stoichiometric by addition of pyruvate and an excess of lactic dehydrogenase, whereby the reduced TPN was continuously removed by its reoxidation. Similar enzymes occur in other animal tissues (27, 28).

The first-formed ribulose-5-phosphate is transformed into an equilibrium mixture containing some 25 % of the 2-ketopentose-5-phosphate (25) by the presence of an enzyme, phosphopentose isomerase, which is not completely removed from the above partially purified dehydrogenases. This isomerase has been purified from alfalfa leaves by Axelrod (29) and is poisoned by mercurials (30); the enzyme from muscle behaves similarly and is completely reactivated by cysteine (16). Hence, as is probable for the two dehydrogenases of this route (31), this appears to be a sulphydryl enzyme which is insensitive to iodoacetate. It is not yet clear if ribulose-5-phosphate is the sole ketopentose formed in this reaction, and xylulose phosphate or another ketopentose phosphate may perhaps also be involved (32, 33) at least in some enzyme preparations (16).

In the primary oxidation of glucose-6-phosphate to 6-phosphogluconate, the pyranose ring remains unbroken (34) :

$$\text{D-glucopyranose-6-phosphate} + \text{TPN} \rightleftarrows \text{6-phospho-}\delta\text{-gluconolactone} + \text{TPNH}_2.$$

The subsequent hydrolysis of the lactone is nonenzymic with purified *Zwischenferment* preparations, according to Horecker and Smyrniotis (35); it is rather slow, the half-period of the lactone being about 1½ minute at pH 7.4. The above reaction was studied in reverse by these authors, by the addition of *iso*citric dehydrogenase together with its substrate as a source of regenerated $\text{TPNH}_2$, when the δ-gluconolactone was reduced to glucose-6-phosphate. The γ-lactone was not effective in this reaction as a source of glucose-6-phosphate. The recent demonstration by Brodie and Lipmann (36) of a lactonizing enzyme, present in bacteria, yeast and liver tissue, makes possible this sequence of reactions at a rate which might be sufficient for phosphogluconate reduction *in vivo* to be of some physiological importance. This is especially likely to occur, when, as in higher plants, there is a mechanism for keeping the TPN reduced.

The reversal of the second stage has also been demonstrated (37) by fixation of $^{14}CO_2$ into the open-chain form of phosphogluconate formed by the reaction catalysed by phosphogluconate dehydrogenase :

$$\text{Ribulose-5-phosphate} + {}^{14}CO_2 + \text{TPNH} + H^+ \rightleftarrows \text{1-}^{14}\text{C-6-phosphogluconate} + \text{TPN}^+.$$

This reductive carboxylation is similar in type to that for the *iso*citrate-α-ketoglutarate and malate-pyruvate conversions. Whether a 3-keto-phosphogluconic acid intermediate, as postulated by Horecker (3), actually occurs is still unknown, but it is of interest that all three reactions are TPN-linked and have other points of similarity, including the non-isolation of the intermediate stage. The recent finding of Glock and McLean (38) that TPN in tissues is mainly present in the reduced forms would somewhat favour the possible reversal of this reaction *in vivo*. This follows from the data of Horecker and Smyrnitois (37), when at constant physiological pH and with 5 % $CO_2$ present in the atmosphere, the equilibrium would be such that :

$$\frac{(\text{6 PG})}{(\text{R-5-P})} = 0.0036\,\frac{(\text{TPNH})}{(\text{TPN})}$$

Since Glock and McLean have found ratios of TPNH/TPN around 20 : 1 in several mammalian tissues, it follows that about 8 % of pentose phosphate could be converted to phosphohexonic acid by this route, at equilibrium. As already mentioned, the free acid formed in this reaction would be lactonized, presumably by the gluconolactonase of Brodie and Lipmann (36) before it could be further reduced to glucose-6-phosphate by reversal of the *Zwischenferment* reaction.

*Breakdown of pentose phosphate*

It is doubtful if reversal of the oxidative route could assume biological importance except under rather special circumstances. However hexose can readily be formed from pentose phosphate in a variety of tissues, not by reversal of its oxidative formation, but by non-oxidative cleavage of pentose phosphate by the enzymes 'transketolase' and 'transaldolase'.

The demonstration of the mechanism of this conversion is mainly due to Racker (39-42) and Horecker (43-52) and their colleagues. Ribulose-5-phosphate is rapidly transformed by a yeast enzyme into triose phosphate, but the reaction proceeds in this direction only if ribose-5-phosphate, as well as ribulose-5-phosphate, is present as an 'acceptor aldehyde'. No free 2-carbon fragment can be detected. The enzyme concerned has been called 'transketolase' and has been highly purified (41, 46). It causes the reaction :

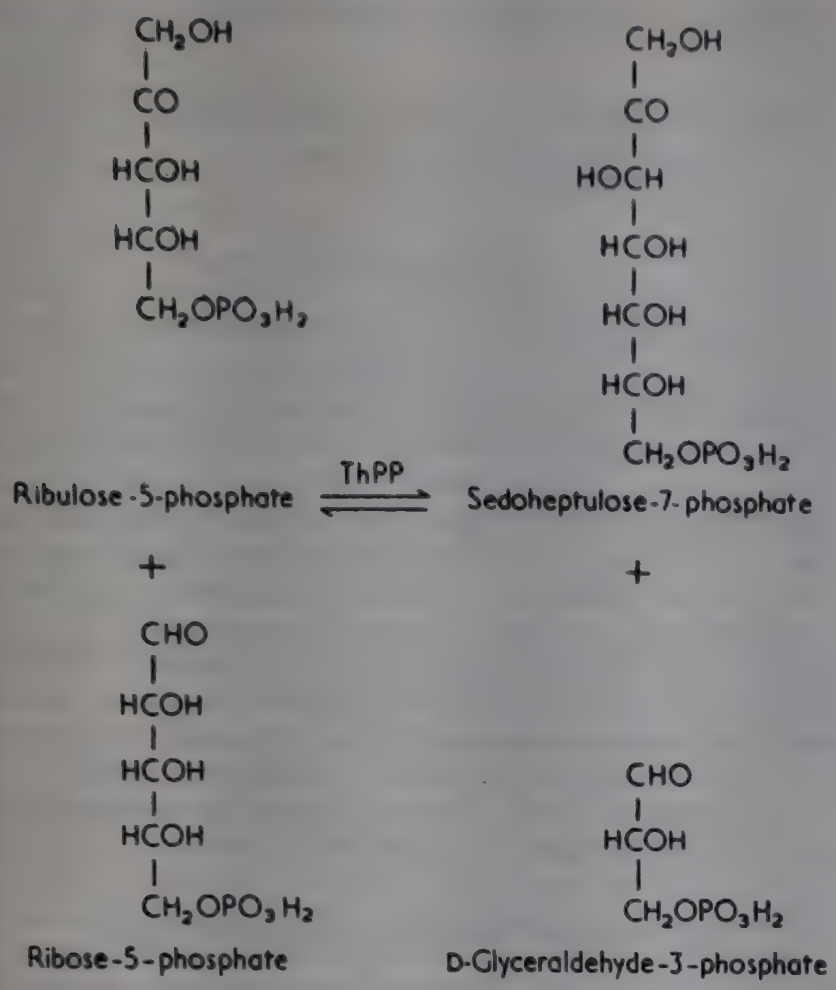


FIG. 3.

or : Ribulose-5-phosphate + Ribose-5-phosphate $\rightleftarrows$ Sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate.

Since the enzyme causes also the transfer of a $C_2$-unit from other compounds, such as hydroxypyruvate, L-erythulose (40, 46) and fructose-6-phosphate (42) to a variety of 'acceptor aldehydes', such as ribose-5-phosphate or glycolaldehyde phosphate (40, 46), it seems to have a wide application in metabolism. The enzyme has been obtained from yeast, liver and spinach leaves : it requires thiamine pyrophosphate and the spinach preparation also requires magnesium ions (46). The action of the coenzyme-enzyme complex may be pictured as accepting a glycolaldehyde residue $CH_2OH\text{-}CO^-$ and transferring it to another aldehyde by a ketol type of condensation :

$$CH_2OH\text{-}CO.... + CHO\text{-}R \rightleftarrows CH_2OH\text{-}CO\text{-}CHOH.R.$$

By the formation of sedoheptulose-7-phosphate in this reaction, a longstanding problem of the biological occurrence of ketoheptoses seems to have been solved. These sugars occur in yeast extracts (53) and higher plants, especially those of the *Sedum* family, but also in other species (54).

A further enzyme system 'transaldolase', transfers the dihydroxyacetone group from the molecule of sedoheptulose to an acceptor aldehyde, *e.g.* 3-phosphoglyceraldehyde, forming fructose-6-phosphate and leaving a tetrose-4-phosphate :

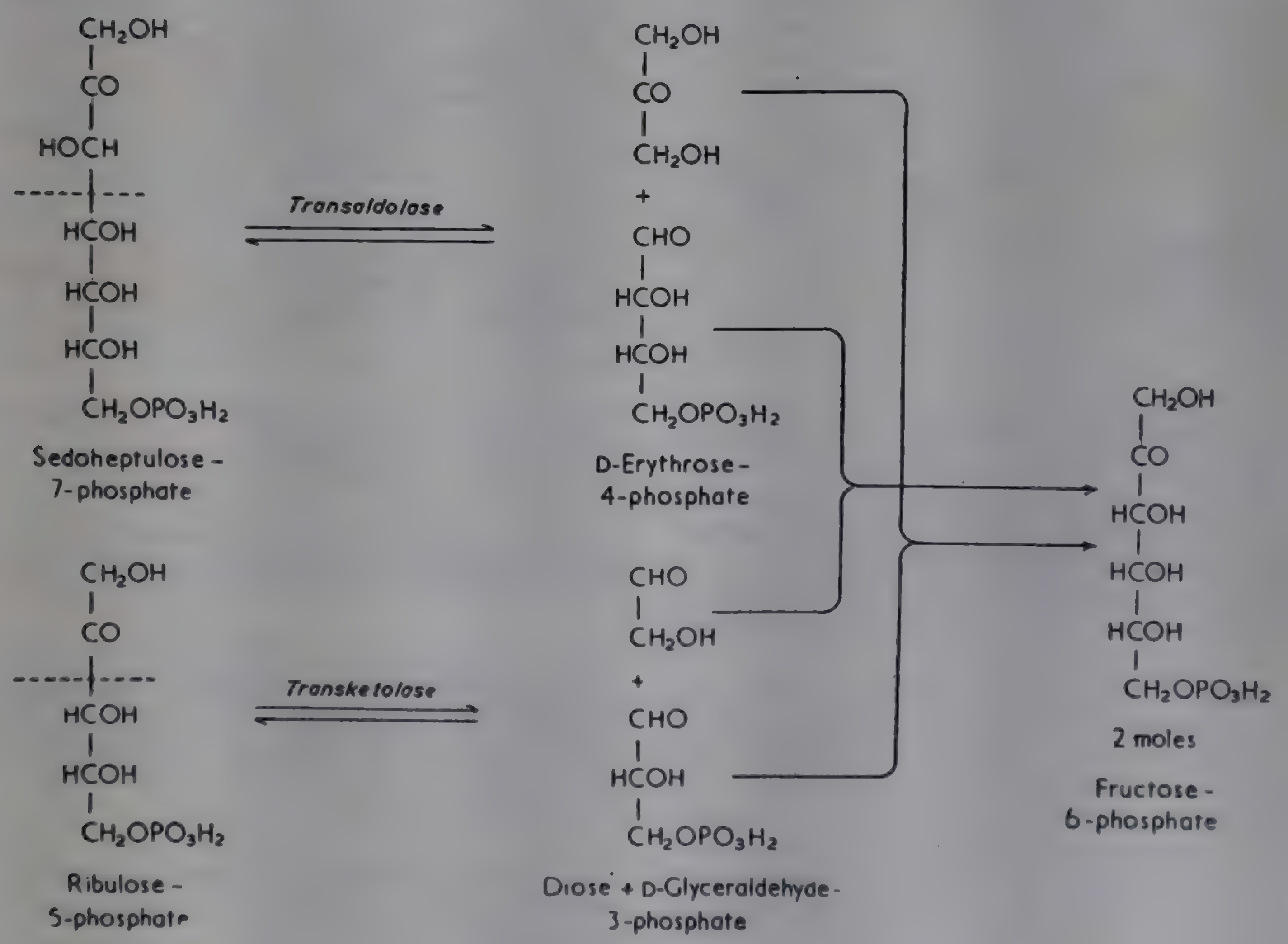


FIG. 4.

The reaction appears to be reversible, with an equilibrium constant of 0.82 at 25° C. So far there is no evidence of a prosthetic group or co-factor being involved in the purified enzyme from yeast (51). The existence of this reaction explains how, as the keto-heptose disappears, the formation of a hexose can be detected, the first product being fructose-6-phosphate (55).

In this way (see figure 4) the molar yield of hexose-phosphate exceeds that which could be formed from only the triose moiety of ribose-5-phosphate, by a simple aldolase condensation. This type of condensation does, however, provide an alternative route of resynthesis of hexose (48).

In the original experiments of Dickens (17), as already mentioned, ribose-5-phosphate was shown to yield with a dialysed yeast enzyme preparation, together with $Mg^{++}$ and DPN, the following reaction :

$$\text{1 mol. Ribose-5-phosphate} \rightarrow \text{1 mol. } C_2H_5OH + \text{1 mol. } H_3PO_4 + \text{1 mol. } CO_2 + C_2H_4O_2$$

The 'diose' residue could not be identified, but its production was inferred from the requirements of the above reaction.

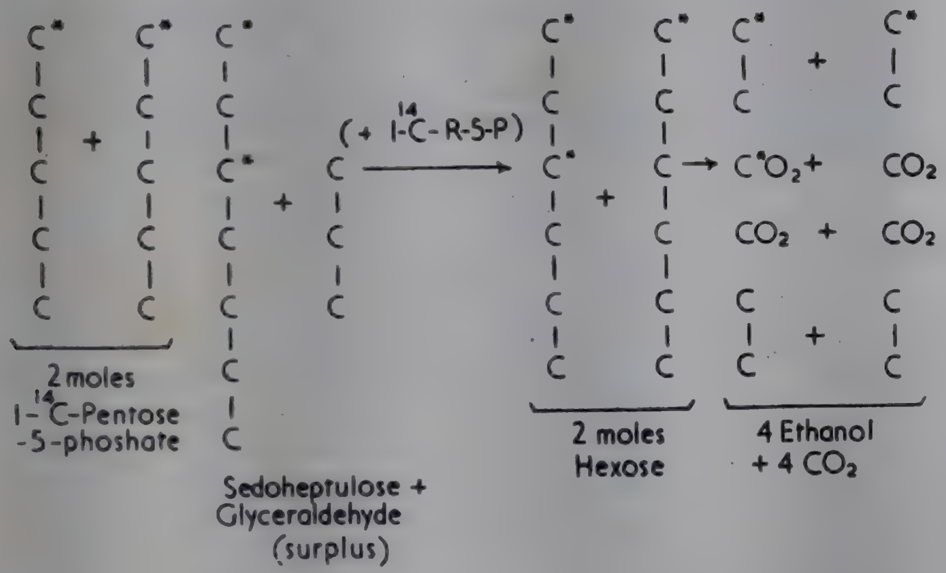


FIG. 5.

Gibbs and his colleagues have recently re-investigated this reaction in similar yeast extracts but with isotopically labelled 1-$^{14}$C-ribose-5-phosphate. They find that, instead of a straightforward cleavage of the pentose into 'diose' and triose phosphate, with fermentation of the latter, the reaction seems to involve transformation of pentose to hexose before the latter undergoes alcoholic fermentation.

As will be seen from figure 2*b*, the direct cleavage, into a diose and a triose, of 1-$^{14}$C-ribose-5-phosphate would result in unlabelled $CO_2$ and ethanol, formed from the triose phosphate by fermentation, while the isotope would be confined to the aldehyde group of 'glycol-aldehyde'. In yeast extracts, however, this does not occur. Recent work of Gibbs, Earl and Ritchie (56) indicates that even in this case the reaction proceeds *via* transketolase and transaldolase (figure 5). The hexose-6-phosphate formed from the pentose *via* heptulose phosphate is the actual substrate for the fermentative breakdown. The expected isotope distribution would then be :

$$\text{2 mol. 1-}^{14}\text{C-R-5-P} \rightarrow \text{1.3-}^{14}\text{C-sedoheptulose} + \text{triose}$$
$$\rightarrow \textbf{1.3-}^{14}\textbf{C-hexose} + \text{tetrose}$$
$$(+ \text{1-}^{14}\text{C-R-5-P}) \downarrow$$
$$\textbf{1-}^{14}\textbf{C-hexose} + \text{triose}$$

Thus the resulting hexose (bold) would be labelled in both 1-C and 3-C with double the activity in the former position. On fermentation of the hexose formed in this way, the resulting $CO_2$ would have 0.25 × the specific activity of the ribose, while the methyl group of the alcohol would be 0.5 × that of the ribose. The observed values of 0.25 and 0.4 indicate that this route is similar to the corresponding anaerobic part of the oxidative phosphorylative pathway already described.

Under the above conditions, no direct oxidative formation of $^{14}CO_2$ from 1-$^{14}$C-glucose occurred, corresponding with the same observation in anaerobic fermentation of 1-$^{14}$C-glucose by intact yeast cells (57).

I am indebted to Dr. Martin Gibbs for permission to quote this work before publication.

*The complete hexosemonophosphate cycle*

The following chain of enzymes acting on glucose-6-phosphate are involved :

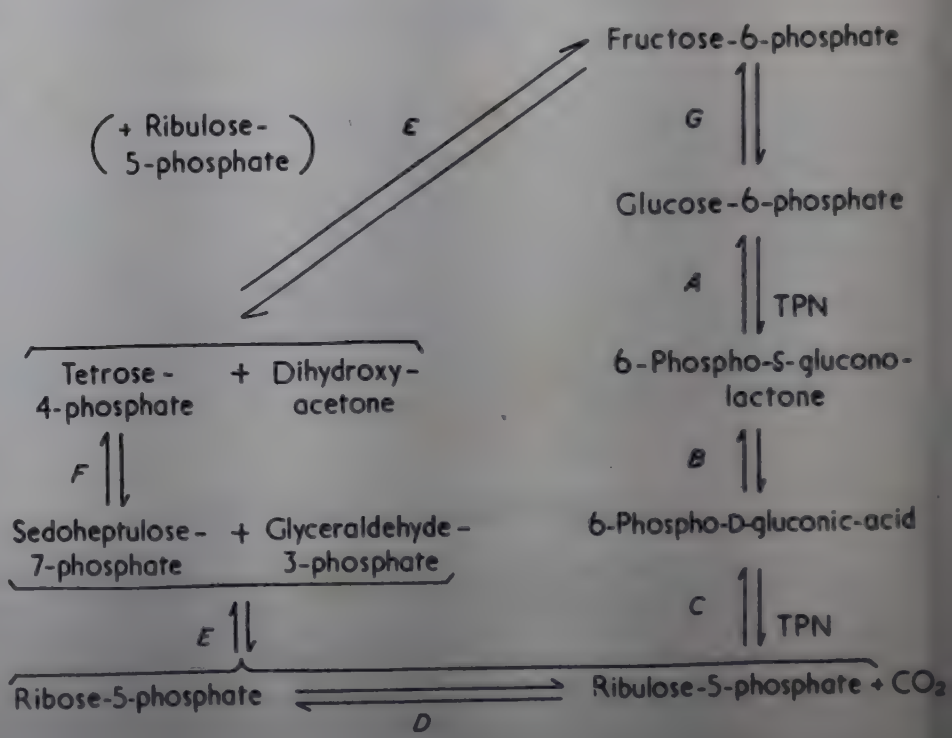


FIG. 6.

(*A*) glucose-6-phosphate dehydrogenase, (*B*) gluconolactonase, (*C*) 6-phosphogluconic acid dehydrogenase, (*D*) pentosephosphate isomerase, (*E*) transketolase, (*F*) transaldolase, (*G*) hexosephosphate isomerase, (*H*) aldolase, (*I*) a fructose diphosphatase (Mg activated, see 48).

The reaction sequence shown in figure 6 is only one of several possible, but its individual reactions all have experimental support.

The main features of this pathway are : (*a*) two oxidative stages, each coupled with TPN, including loss of

carbon atom 1 of glucose as $CO_2$ and formation of ribulose-5-phosphate.

(*b*) Formation of pentose-ketopentose phosphate equilibrium mixture.

(*c*) Transketolation of 'active glycolaldehyde' from ribulose to a ribulose molecule.

(*d*) Cleavage of dihydroxyacetone by transaldolase from the sedoheptulose formed in (*c*), leaving a tetrose phosphate and its transfer to a triose phosphate molecule, giving fructose-6-phosphate.

(*e*) Transfer of another 'active glycolaldehyde' unit from a third molecule of ribulose to the tetrose, yielding another fructose-6-phosphate molecule.

(*f*) Formation of Robison equilibrium ester by phosphohexose isomerase.

(*g*) The overall reaction is therefore :

$$3 \text{ Glucose-6-phosphate} + 3\ O_2 \rightarrow 2 \text{ Fructose-6-phosphate} + 1 \text{ Triose phosphate} + 3CO_2 + 3\ H_2O$$

in which, by each turn of the cycle, one triose equivalent is completely oxidized, with an R.Q. of unity. In intact cells, the triose phosphate accumulating could of course be disposed of by triose phosphate isomerase and aldolase, yielding fructose phosphate again, and thus either entering the glycolytic chain or, after conversion to monophosphate, re-entering the HMP pathway. In the latter case, no stage below triose phosphate need be involved.

The identification of the tetrose phosphate as D-erythrose-4-phosphate is at present still tentative, as this compound has not been accumulated in amount sufficient for identification (52). However, chromatographic evidence, and the condensation with triose phosphate in presence of muscle aldolase to form a heptulose-diphosphate having one acid-labile phosphate, supports the formation of this tetrose ester (52). Yeast transaldolase has been purified and seems to be more selective than transketolase, the only donors of dihydroxyacetone yet found active being sedoheptulose-7-phosphate and fructose-6-phosphate (51). There can be no doubt that the discovery of the mechanism of these two enzymes is of first-rate importance for biochemistry.

### *The distribution of isotopic carbon among the reaction products*

There is some difference, according to the source of the extracts, in the distribution of isotopic carbon atoms derived from ribose-5-phosphate labelled either $1\text{-}^{14}C$ or $2,3\text{-}^{14}C$.

Liver preparations yielded hexosemonophosphate from both types of labelled pentose according to the scheme already mentioned, with the additional conversion of the final triose phosphate by aldolase to hexose (48). For instance, the $1\text{-}^{14}C$-pentose gave 74 % of the total radioactivity in 1-C of the isolated glucose-6-phosphate, and 24 % in 3-C. With a pea root preparation (50) the figures were about the same, but the pea leaves gave evidence of some further mechanism whereby 4-C and 5-C of the hexose chain also received some isotope from $1\text{-}^{14}C$ of ribose. Spinach extracts, in the dark, can fix $CO_2$ into phosphoglyceric acid, which appears to arise *via* carboxylation of ribulose diphosphate (49, 57). This important reaction has a vital bearing on problems of photo-synthesis to be discussed by other speakers today.

### *Distribution of isotopic carbon with intact cells*

Theoretically it should be easy to distinguish the glycolytic from the hexose monophosphate pathway by comparison of the liberation of $^{14}CO_2$ when $1\text{-}^{14}C$-glucose and $6\text{-}^{14}C$-glucose are the substrates. In the glycolytic pathway, exclusively carbon atoms 3 and 4 of the glucose chain are expected to yield $CO_2$, whereas in simple loss of carbon atom 1, obviously $1\text{-}^{14}C$-glucose should be the sole contributor to the isotopic $CO_2$. The fact that the tissues own carbohydrate reserves are also metabolised can be compensated by comparison with glucose labelled in other positions (60). But a more serious difficulty is the existence of re-cycling of pentose formed by decarboxylation giving hexose, which would again tend to yield further quantities of carbon dioxide without any obligatory passage through glycolysis (figure 6). Other quite different metabolic pathways may also occur (see 5). The existence of these difficulties probably explains different conclusions even from almost identical experimental data (58, 59), in all of which calculations certain hypothetical assumptions were made. Agranoff, Brady and Colodzin (60) have reviewed the difficulties and advise against a quantitative evaluation on present data. On the other hand Bloom and Stetten (61), who have recently radically revised their original calculations, now conclude that quantitative assessment is possible. Incubating liver slices with glucose-$1\text{-}^{14}C$, $-2\text{-}^{14}C$ and $-6\text{-}^{14}C$ and applying these equations, they find that half the glucose metabolized is glycolysed and the remainder is oxidized by the hexosemonophosphate pathway. In diaphragm, on the other hand, the whole is utilized via glycolysis. In liver slices, labelled gluconate probably entered the oxidative pathway also. These experimental results are supportered by those of Agranoff *et al.* (60), who observed that dietary effects were evident : the 6-C/1-C ratio was raised in fasting animals, as well as in regenerating, fœtal and malignant liver tissue. Whereas this ratio was about 0.3 in normal rat liver it was near unity in kidney and diaphragm. Here a similar result was also obtained by Katz *et al.* (59).

In their study of the distribution of the enzymes of the monophosphate pathway in animal tissues, Glock and McLean (62, 63) have observed a very great increase of the activity in the mammary gland of the rat during lactation. Abraham, Hirsch and Chaikoff (64) have followed this up by a study of the utilization of $1\text{-}^{14}C$, $2\text{-}^{14}C$, $3,4\text{-}^{14}C$, $6\text{-}^{14}C$ and (evenly) $E\text{-}^{14}C$ labelled glucose, together with its incorporation into fats. $1\text{-}^{14}C$ glucose yielded $CO_2$ at more than 10 times the rate of $6\text{-}^{14}C$-glucose, while the lipid-$^{14}C$ recoveries were more than twice as great with $6\text{-}^{14}C$-glucose as substrate. It was concluded that at least 60 % of the glucose molecules in the lactating mammary gland of the rat were utilized by a 'direct' oxidative pathway. This strongly supports the value of determination of enzymes levels, at least as a preliminary study.

In the ocular lens, the ratio $1\text{-}^{14}C/6\text{-}^{14}C$ of glucose converted to $CO_2$ may reach 40 : 1 (Kinoshita, 65).

This would indicate a predominantly HMP-pathway. In corneal epithelium the HMP-pathway seems to be coupled via TPN with glutathione reductase, thereby keeping a level of reduced glutathione protective to eye-tissues. The interesting suggestion has been made (66) that, by avoiding the formation of lactic acid, the HMP-pathway may further shield these avascular tissues from the injurious effects of this acid.

In the adrenal gland Glock and McLean (63) first showed the very active nature of the HMP-enzymes. This has been recently confirmed by Kelly *et al.* (67), who suggest a possible connexion with the TPN catalysed β-11-hydroxylation of steroid hormones.

Other animal tissues having high activity in the HMP-enzymes are bone marrow (27) kidney, liver and liver carcinoma (28), heart, ovary and particularly the adrenal gland (63). Figure 7*a* brings out the interesting points that (*a*) adult tissues of high glycolytic activity (as measured by $Q^{N_2}_{CO_2}$) tend to have also high hexokinase contents, taking the latter from the data of Long (68); (*b*) there is a rough inverse relationship between these values and the distribution of the hexose monophosphate enzymes, taken from the data of Glock and McLean (63); (*c*) figure 7*b* shows that embryonic, malignant, proliferating and some hormone-dependent tissues (lactating mammary gland, ovary, adrenal) tend to have high activity in the HMP pathway combined with high glycolysis. It is also noteworthy that highly glycolysing normal tissues usually have a R.Q. near unity, while tumour tissues have a lower value as a rule (69). One might express the overall impression by saying that growing and tumour tissues show a tendency to develop

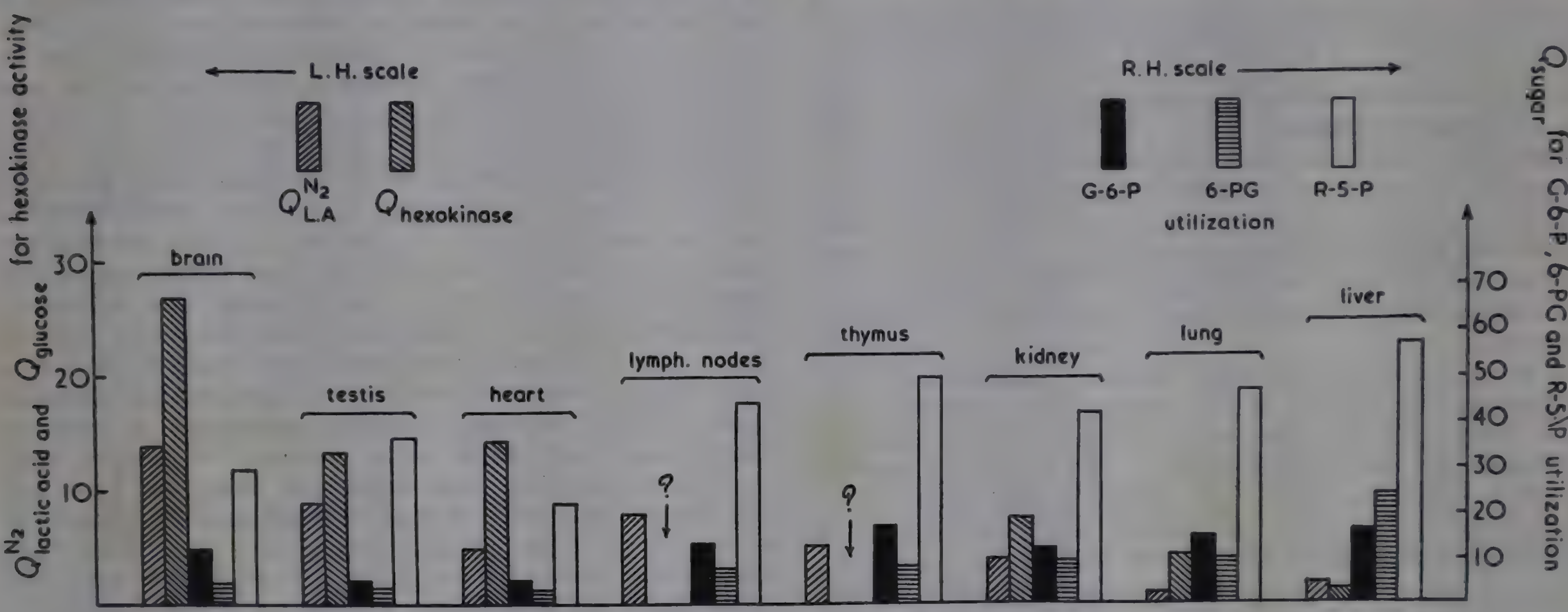


FIG. 7*a*. — Comparison of glycolysis $(Q^{N_2}_{CO_2})$, activity of hexokinase and the enzymes of the HMP-oxidative pathway, n some tissues of the rat. Adult tissues.

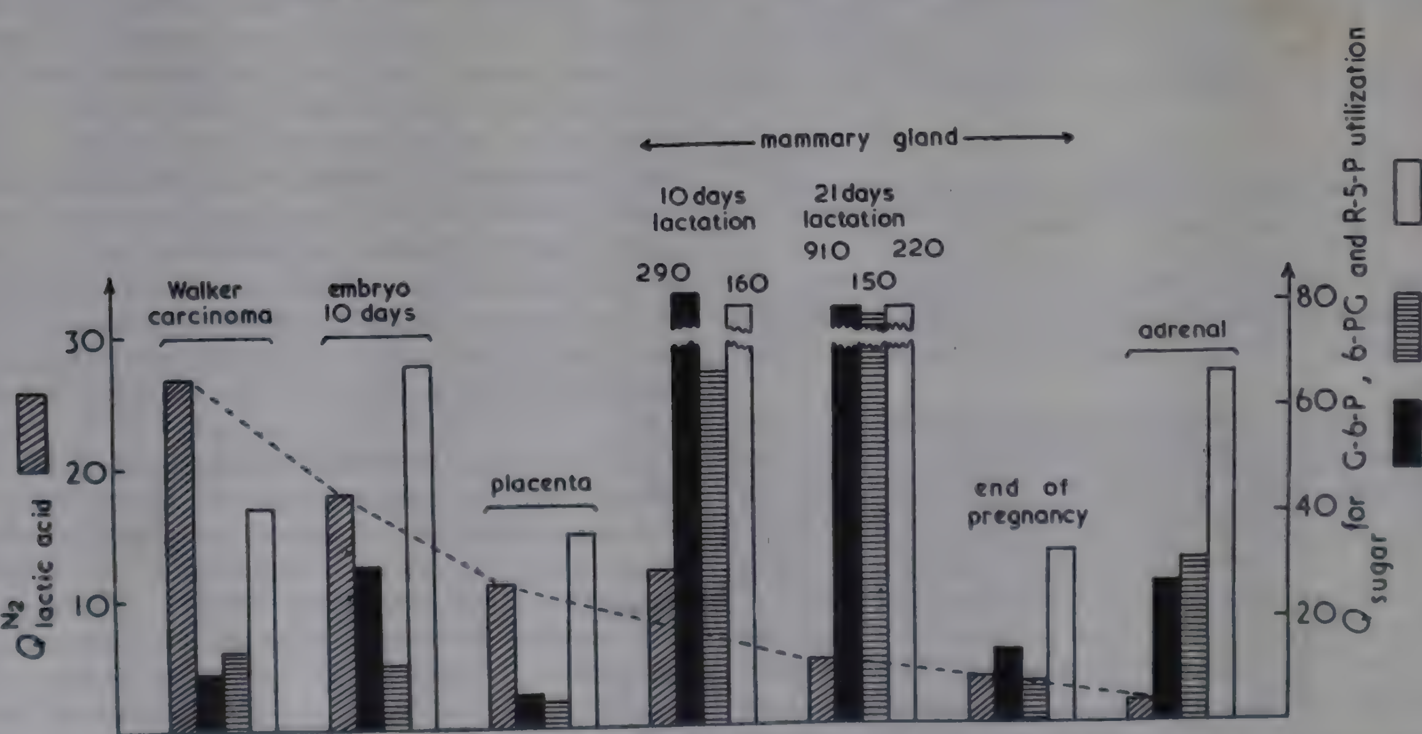


FIG. 7*b*. — Comparison of glycolysis $(Q^{N_2}_{CO_2})$, and the enzymes of the HPM-oxidative pathway, in some tissues of the rat. Growing and hormonally controlled tissues. For data see Glock and McLean (62, 63); Long (68).

both pathways of carbohydrate oxidation, whereas adult tissues vary greatly in their equipment for these two routes, but often seem to have mainly or almost exclusively a glycolytic type of metabolism, especially the more specialized structures such as brain and skeletal muscle. The lower ratio of $CO_2$ derived from 6-$^{14}$C-/1-$^{14}$C glucose in hepatoma transplants (60) compared with normal mouse liver support this view, but as the HMP pathway is in any case high in liver a more increase in glycolytic pathway would suffice for conformity with the above criterion, and could actually result in higher 6-$^{14}$C/1-$^{14}$C ratios. Such ratios are therefore an uncertain index when taken by themselves.

It is tempting to fit these facts to the need for proliferating tissues to synthesise relatively large amounts of RNA, as well as DNA. The former may require HMP oxidation, the latter glycolysis, as in Cohen's experiments with virus-infected *E. coli* (ref. 2, p. 221). Much more work is needed on this subject. However, Glock and McLean have found a close correlation between RNA content and levels of the HMP enzymes in liver tissue of rats under widely varying conditions of nutrition and hormonal balance. While this may merely be a non-specific reflexion of the degree of protein synthesis, it is also possible that a direct relationship between ribose-phosphate formation and RNA synthesis exists.

### *The levels of TPN and DPN in tissues in relation to the HMP oxidative route*

It is known that the amount of DPN exceeds, often considerably, that of TPN in such animal tissues as have been studied hitherto. Comprehensive data of Glock and McLean are now available (70). Total TPN/total DPN ratios of about 0.3-0.1 are reached in comparitively few tissues : for example, liver, adrenal, lactating mammary gland, kidney, ovary. These are also tissues with high levels of TPN-specific HMP oxidative enzymes (63). Brain, diaphragm, skeletal muscle, spleen, pancreas, testis, pancreas blood, thymus and thyroid gland, have very little TPN, either relatively to DPN or absolutely. The obvious assumption would be that this group has predominantly a glycolytic pathway. The ratios of liberation of $^{14}CO_2$ from isotopic glucose support this classification, in the few cases in which they have yet been determined, as already described.

The possibility that a low TPN content might support a considerable TPN oxidation through the action of Kaplan and Colowick's pyridine nucleotide transhydrogenase has to be considered. Dr. G. F. Humphrey (71) has recently studied in our laboratory the distribution of this enzyme and finds it most active in heart, followed by liver > skeletal muscle > kidney > brain > testis > spleen. This distribution does not suggest that participation of this enzyme in tissues of low TPN content would be particularly helpful to the HMP pathway. However, *iso*citric dehydrogenase, generally regarded as a TPN-linked enzyme (but see 72), apparently functions in brain respiration successfully, despite very low TPN levels in brain (73, 70). This may be a question of localization within the cell, the *iso*citric dehydrogenase functioning in the Krebs cycle, predominantly in the mitochondria. This consideration is likely to be less helpful to the enzymes of the HMP pathway, which reside almost entirely in the soluble-enzyme fraction (31).

More puzzling is the large extent to which the TPN extracted from animal cells is in the reduced form, while the converse usually applies to DPN (70). Evidently the systems reoxidizing TPNH are limiting in the cell, and probably this usually means the TPN-cytochrome *c* reductase. In any case the DPN systems cannot be in equilibrium with the TPN ones, assuming that the redox potentials of the two coenzyme systems are approximately equal. These facts may also be connected with spatial separations in particles and cytoplasm.

### *The synthesis of nucleotides and the HMP pathway*

Any attempt at full discussion of this problem is impossible here. Cohen (2) has shown how the HMP pathway could provide all the ribose-5-phosphate needed for growing cells of *E. coli*, even though quantitatively it constitutes much less of cell respiration than the glycolytic pathway. Probably similar considerations apply to mammalian tissues. Experiments showing a lack of incorporation of isotopic acetate or glycine (74, 75) into RNA-ribose are not conclusive because of dilution effects (Racker, 1). The work with isotopically labelled glucose described above could provide better support for a hexosemonophosphate source, but this line of work has not yet been sufficiently explored for a final verdict. Evidence favouring attachment of ribose-phosphate to precursors of purines before ring closure occurs, yielding the mononucleotide, is accumulating; *e.g.* in the biosynthesis of inosinic acid and other purines (76, 77). Another probable route is the attachment of ribose in nucleotides, due to the action of nucleotide phosphorylase on ribose-1-phosphate and the purine, with subsequent phosphorylation of the ribotide by a specific phosphokinase. The conversion of ribose-5-phosphate, formed in the HMP pathway, to ribose-1-phosphate could occur by the enzyme phosphoribomutase, perhaps identical with phosphoglucomutase (78), which has been described in cell extracts. Other alternatives are condensation of purine with ribose-1.5-diphosphate (78), or step-wise production by the combination of 2-carbon and 3-carbon units in attachment with the purine, similarly to that suggested as a step in the synthesis of DNA by Racker (79). These various possibilities need much fuller investigation at the present time.

### *Hormonal control of the oxidative pathway*

It was tentatively suggested (80) that the existence of alternative pathways could be an important mechanism of hormonal control of metabolism, if hormones were able to regulate which of the routes was taken by foodstuffs. In our laboratory, Drs. Glock and McLean have now obtained some support for this idea. The sex difference in activity of enzymes of the HMP pathway in rat liver is strinkingly in favour of the female (63), and the great increase in amount of these enzymes in lactating mammary gland (63) has already been described. The latter effect is accompanied by some increase

in the same enzymes in the liver. Observations of hormonal effects in liver need careful pair-fed controls, because restriction of food intake itself markedly lowers the amount of glucose-6-phosphate and 6-phosphogluconate dehydrogenases in liver, the amount of RNA falling simultaneously. With this precaution, Glock and McLean have found (81) that in alloxan diabetes the levels of both dehydrogenases are reduced to only one-third of those in the control animals and this effect is also paralleled by a fall in RNA content. Other hormones are now being studied in this respect (82).

### *Other pathways of carbohydrate metabolism*

Many cells, especially micro-organisms, evidently possess either modified or else quite different pathways from those discussed here (1, 2, 5) some of which do not appear to involve phosphorylation of the substrate, thought they eventually presumably provide energy for phosphorylation of ATP.

It must also be remembered that intermediates of the HMP pathway may have functions other than the formation of RNA precursors in the cell. Two recent suggestions are that ribose-5-phosphate may be a histidine precursor in *Neurospora* (83) and a purine nucleotide precursor in liver tissue, as already mentioned (77). The discovery of such new routes of metabolism usually follows soon after a main pathway has been opened up, and in this respect the hexosemonophosphate oxidative pathway may prove to be a stimulus to future work, in the same way as the Embden-Meyerhof pathway has been, and indeed still is.

### *The HMP pathway in micro-organisms and in photosynthesis*

Since these subjects will form the basis of separate contributions to this symposium, it is only necessary here to point out that although mainly only animal tissues have been discussed so far, the applications to other forms of living cells, especially to micro-organisms and photosynthetic and other plant tissues, are at least as important. Indeed, as Calvin and his colleagues have shown, the enzymes and intermediate compounds of the hexosemonophosphate pathway are extraordinarily active in the leaves of higher plants, and for the first time a reductive synthesis of carbohydrate from carbon dioxide in plant extracts has been achieved very recently by Racker (33). These important aspects will be developed by the subsequent contributors to this symposium.

The literature of this subject grows so quickly that the above represents only a fraction of the excellent research in active progress in this field, and many important contributors have necessarily been omitted from this condensed account.

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# Some aspects of carbohydrate oxidation in 'Pseudomonas fluorescens' and 'Microbacterium lacticum'


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(*Received* 1 *August* 1955)

The excellent presentation by Doctor Dickens emphasizes the complicated mosaic of reactions which take part in carbohydrate metabolism in yeast and animal tissues. It is noteworthy although not unexpected in this era of comparative biochemistry that many of the same concepts apply to bacteria. Yet with the great diversity of bacterial types available for study it is not strange that some organisms should be found which differ radically in certain aspects of their carbohydrate catabolism. By way of illustration I should like to present a summary of our studies with two widely different organisms, *Pseudomonas fluorescens* an obligate aerobe and *Microbacterium lacticum* a facultative aerobe. In *Pseudomonas fluorescens*, which does not possess an intact glycolytic scheme, evidence has been found for several pathways of oxidation which convert hexose ultimately to pyruvate as does the classical glycolytic system in other cells (1). These pathways are shown in figure 1. In the absence of ATP, glucose is completely oxidized in 2 steps to 2-ketogluconate (2). These oxidations are also quantitatively important in growing cultures and are considered to furnish energy through oxidative phosphorylation. The complete system which appears to utilize cytochromes *b* and *c* rather than the

flavoprotein or pyridine nucleotide carriers of glucose oxidase and glucose dehydrogenase, resides in a small unit or particle (1).

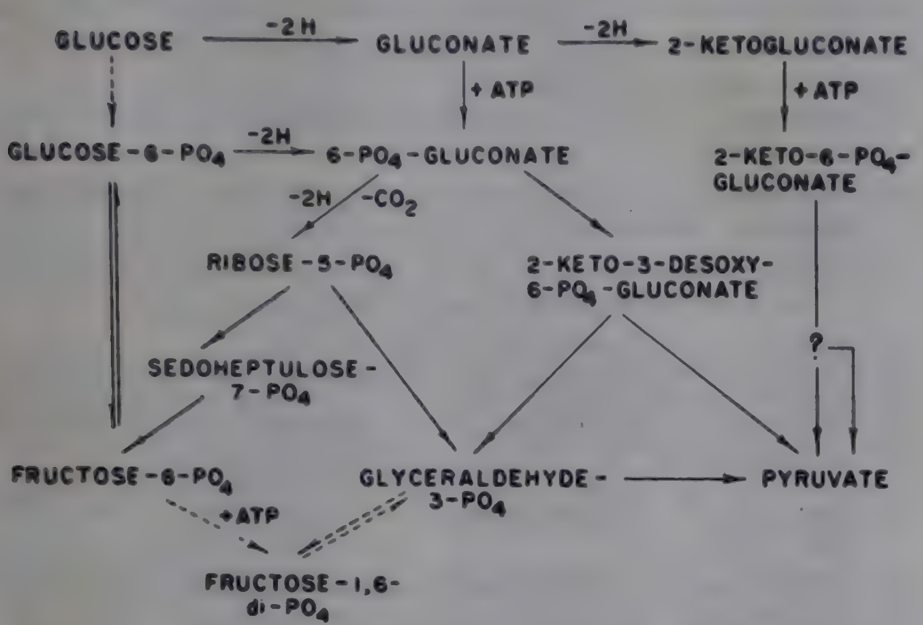


FIG. 1. — Pathways in glucose oxidation (*P. fluorescens*).

The next reaction is a phosphorylation of 2-keto-gluconate (3, 4) to 2-keto-6-phosphogluconate. 2-Keto 6-phosphogluconate is then converted to 2 moles of pyruvate by an undefined and presumably new pathway (5). A detailed discussion of 2-ketogluconate metabolism will be presented by Doctor De Ley. Thus one pathway of glucose catabolism, which is shown along the top and right hand side of figure 1, forms 2 moles of pyruvate as in glycolysis.

Alternate routes begin with the phosphorylation of gluconate (3). The 6-phosphogluconate so formed may then undergo dehydration between carbon atoms 2 and 3 to form 2-keto-3-deoxy-6-phosphogluconate (6) which is then cleaved to give pyruvate and D-glyceraldehyde-3-phosphate (7). Since glyceraldehyde-3-phosphate is degraded with the formation of pyruvate (8), this route also yields 2 equivalents of pyruvate.

Alternatively 6-phosphogluconate may be oxidized (9), presumably to ribulose-5-phosphate. Added ribose-5-phosphate yields successively sedoheptulose phosphate, fructose-6-phosphate and glucose-6-phosphate (9) as described by Doctor Dickens for yeast, animal and plant preparations. Since glucose-6-phosphate dehydrogenase (9) is also present, all of the enzymes for a cyclic oxidation of hexose monophosphate have been demonstrated.

Of the glycolytic pathway to pyruvate shown at the left, only phosphohexokinase and enzymes for the conversion of glyceraldehyde-3-phosphate to pyruvate have been found in quantity (8, 9). Repeated attempts to demonstrate hexokinase and phosphohexokinase have been negative; in addition, aldolase activity is weak. Thus those enzymes which are present may be considered to function as part of the hexose monophosphate pathways. In this organism, therefore, fructose-1,6-diphosphate appears to be the only glycolytic intermediate for which we do not know a function.

In contrast, *Microbacterium lacticum* possesses an intact glycolytic scheme and a yeast-type hexose monophosphate pathway (10). Evidence has not been obtained for oxidations prior to phosphorylation which yield gluconate or ketogluconate such as found in *P. fluorescens*, or for the pathway of 6-phosphogluconate cleavage discovered in *P. saccharophila* by Entner and Doudoroff (11) and also found in *P. fluorescens* (6, 7). Under anaerobic conditions, ribose-5-phosphate yields successively heptulose and hexose monophosphate as has been demonstrated in many cells. This sequence can also be duplicated under aerobic conditions with preparations made deficient in DPN and TPN by charcoal treatment (10) (figure 2). With DPN added or with untreated extracts,

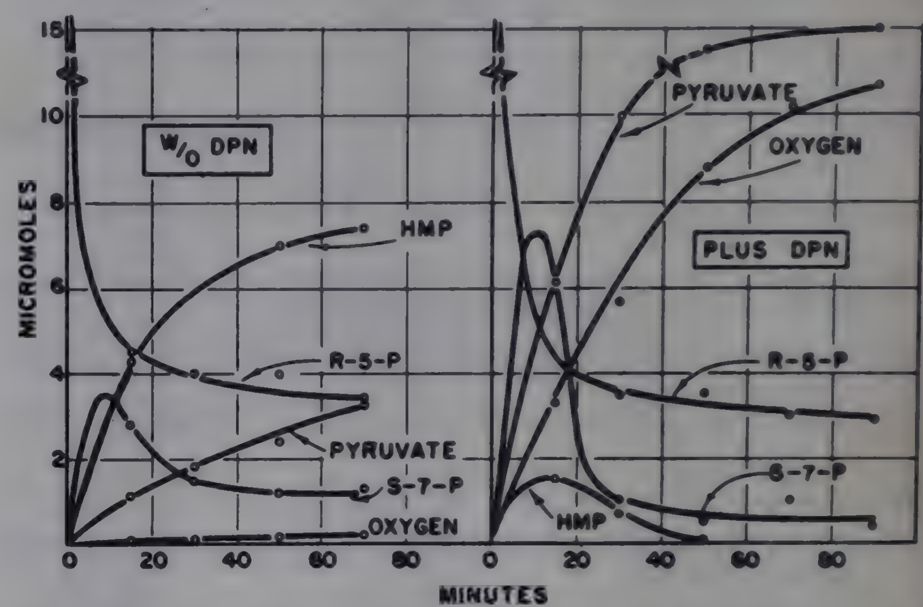


FIG. 2. — Ribose-5-P degradation; effect of DPN (Darco treated *M. lacticum* extract).

however, aerobic ribose-5-phosphate degradation is accompanied by oxygen consumption and pyruvate formation. Furthermore, heptulose and hexose monophosphate are formed initially but soon disappear. Carbon dioxide is not evolved (12). Thus under aerobic conditions, even though the enzymes of the hexose monophosphate cycle, DPN and TPN are present, this pathway does not assume major importance. Instead another pathway which presumably yields triosephosphate predominates. Evidently glyceraldehyde-3-phosphate formed by ribulose-5-phosphate cleavage is preferentially oxidized rather than serving as a substrate for transaldolase. If transketolase were the sole enzyme for cleaving the ribulose carbon chain, then the oxidation of glyceraldehyde-3-phosphate would cause the accumulation of sedoheptulose-7-phosphate. This does not occur and further, added sedoheptulose-7-phosphate is utilized very slowly.

Four pathways are known by which pyruvate can be formed from ribose-5-phosphate as shown in figure 3. Ribulose-5-phosphate may be converted to fructose-6-phosphate by the action of transaldolase and transketolase. Fructose-6-phosphate may then be degradated *via* (*a*) the glycolytic route to pyruvate as shown at the left, (*b*) *via* the hexose monophosphate cycle, or (*c*) oxidized to 6-phosphogluconate which then gives rise to pyruvate and glyceraldehyde-3-phosphate *via* 2-keto-3-deoxy-6-phosphogluconate. The fourth pathway, presumably not involving transketolase and transaldolase

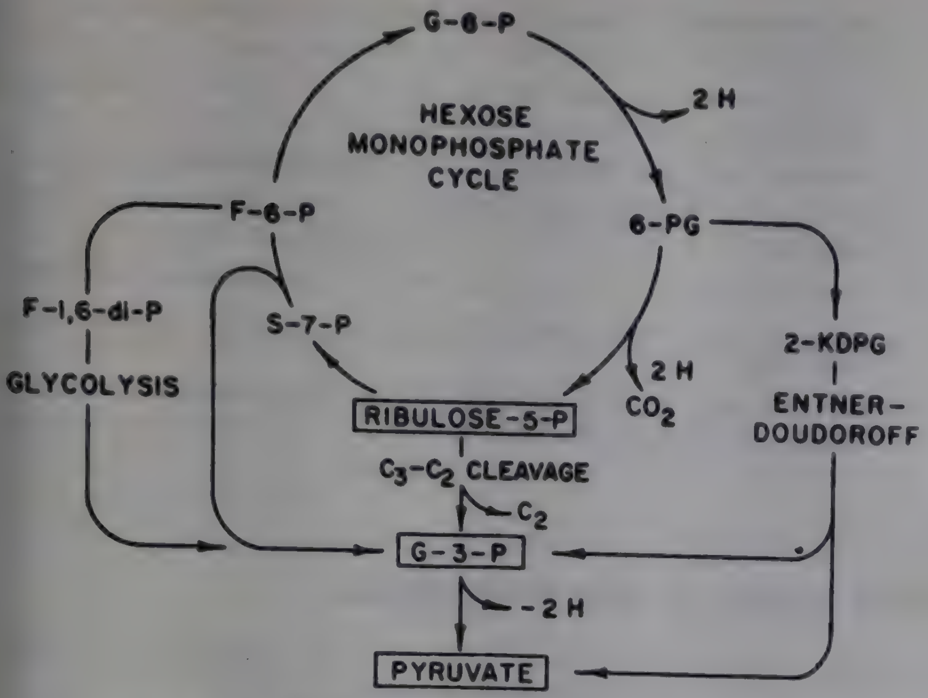


FIG. 3. — Pathways from pentose to pyruvate.

utilizes a cleavage to free 2 and 3 carbon units with the 3 carbon unit serving as a precursor of pyruvate.

The stoichiometry expected for conversion to pyruvate solely by one of these routes is shown in table I. It can be seen that *via* the hexose monophosphate cycle, per mole of ribose-5-phosphate utilized, 1 mole pyruvate

TABLE I

*Stoichiometry for conversion of ribose-5-phosphate to pyruvate by alternate pathways*

| Pathway | Pentose | $O_2$ | Pyr | $CO_2$ |
|---|---|---|---|---|
| Hexose monophosphate cycle . . . . . . . | 1 | 2.5 | 1 | 2 |
| *Via* HMP and F-1, 6-P (Glycolysis) . . . | 1 | 0.83 | 1.67 | 0 |
| *Via* HMP and 6-PG cleavage . . . . . | 1 | 0.83 | 1.67 | 0 |
| 3-2 Cleavage of pentose | 1 | 0.5 | 1 | 0 |
| *M. lacticum* . . . . . | 10.4<br>1 | 5.3<br>0.5 | 9.4<br>0.9 | 0.8<br>0.1 |

and 2 moles carbon dioxide are produced. Carbon dioxide is not produced by the other pathways, but 1.67 moles of pyruvate are formed *via* either route involving fructose-6-phosphate. The direct cleavage yields 1 mole of pyruvate but not carbon dioxide with carbon atoms 1 and 2 being unaccounted for. The typically observed stoichiometry of ribose-5-phosphate degradation closely follows that shown by the fourth line, *i.e.*, 10 µ-moles of ribose-5-phosphate yielded 9 µ-moles of pyruvate and 1 µ-mole of carbon dioxide, and consumed 5 µ-moles of oxygen (12).

These pathways can also be clearly differentiated by using position-labeled substrates as shown in table II. For instance if a 3-2 cleavage rather than one of the other pathways involving transketolase were occurring, pyru-

TABLE II

*Distribution of $^{14}C$ in products from R-5-P-$^{14}C$*

| Pathway | R-5-P-1-$^{14}C$ | | R-5-P-2,3-$^{14}C$ | |
|---|---|---|---|---|
| | $^{14}C$-Product | Position | $^{14}C$-Product | Position |
| 1. HMP cycle . | $CO_2$ | — | $CO_2$<br>pyruvate | —<br>carboxyl |
| 2. *Via* HMP, F-1,6-P (glycolysis) | pyruvate | methyl<br>carboxyl | pyruvate | carboxyl<br>carbonyl |
| 3. *Via* HMP and 6-PG cleavage . . . | pyruvate | carboxyl<br>methyl | pyruvate | carboxyl<br>carbonyl<br>methyl |
| 4. 3-2 Pentose split . . . | $C_2$ unit | — | $C_2$ unit<br>pyruvate | carboxyl |
| 5. *M. lacticum* | pyruvate | total 1.0 % | pyruvate | COOH 60 %<br>C =0 11 %<br>$CH_3$ 7 % |

vate produced from ribose-5-phosphate-1-$^{14}C$ would be unlabeled whereas that from ribose-5-phosphate-2,3-$^{14}C_2$ would contain $^{14}C$ in the carboxyl group. In contrast, by the routes involving fructose-6-phosphate as an intermediate, pyruvate from ribose-5-phosphate-1-$^{14}C$ would be labeled or $^{14}CO_2$ would be formed. With ribose-5-phosphate-2,3-$^{14}C$ pyruvate would be labeled in more than one position.

When these substrates were degraded by *M. lacticum* extracts, the pyruvate isolated with ribose-5-phosphate-1-$^{14}C$ as the substrate was unlabeled whereas that from ribose-5-phosphate-2,3-$^{14}C_2$ contained 60 % of the specific activity of carbon atom 2 or 3 of ribose-5-phosphate in the carboxyl group. Carbon dioxide was not produced (12). Since this labeling can occur by only one of the four pathways described, *i. e.*, cleavage to yield 3 and 2 carbon units (figure 4), we are led to conclude

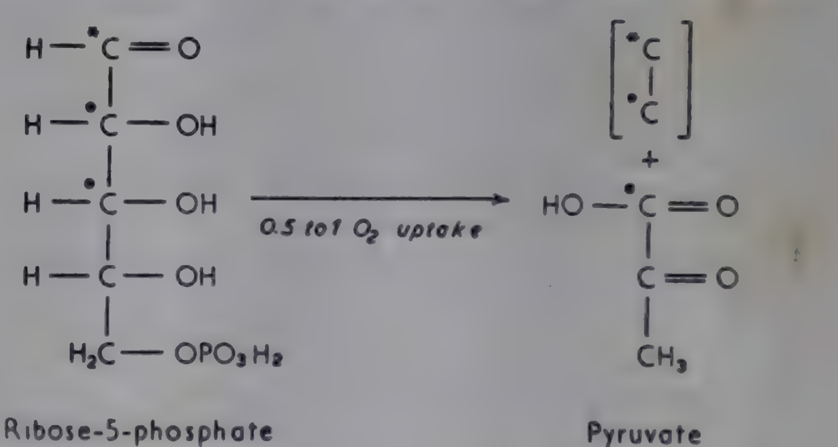


FIG. 4. — Oxidation of ribose-5-phosphate (*M. lacticum*).

that although the enzymes of the hexose monophosphate cycle are present, a cleavage for ribulose-5-phosphate possibly like that shown in figure 4 is of major importance under aerobic conditions. The nature of this cleavage is unknown for the missing carbons 1 and 2 of ribose-5-phosphate have not yet been found.

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# The hexosemonophosphate oxidative route in micro-organisms

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(*Received* 23 *July* 1955)

The enzymes of the hexose monophosphate (HMP) oxidative route are present in some animal tissues, higher plants, yeasts, and some other micro-organisms. It is the aim of this report to review briefly our present knowledge on the presence of this pathway in the carbohydrate metabolism of micro-organisms. The prototype of this pathway in yeast, *Saccharomyces cerevisiae*, has been discussed at length in the preceding lecture and will not be considered further here.

*The HMP oxidative route in the Enterobacteriaceae*

This family has been investigated best of all the bacteria. Two members of this family have been particularly well studied : *Escherichia coli* and *Aerobacter cloacae*. The formation of pentose phosphates from glucose-6-phosphate (G-6-P) in the former bacteria has been thoroughly studied by Cohen and his collaborators (1-9). Several other authors have elucidated other reactions of this pathway (10-13). *A. cloacae* has been investigated in our laboratory (14-19). It appears that this mechanism is entirely analogous to the pathway in yeast and higher tissues.

These bacteria possess in addition a number of adaptive kinases, which enable them to grow on several unusual substrates : gluconokinase (1,3, 17), 2-ketogluconokinase (17), ribulokinase (20) and ribokinase (2, 7, 17, 21). In *E. coli* D-arabinose is transformed into D-ribulose by an adaptive pentose isomerase (20). This enzyme also catalyzes the conversion of L-fucose into L-fuculose (22). It is very probable that desoxyribose (23) and sedoheptulose (24) also enter the metabolism after a previous transformation into the respective phosphoric esters. Several other members of this family grow also very well on sedoheptulose (25). It is for the moment not yet sure if the metabolism of D-xylose (and of D-lyxose and D-arabinose) is really connected with the HMP oxidative route. With one strain of *E. coli* (12), D-xylose yields 0.8 mole 3-phosphoglyceric acid, indicating a $C_2$-$C_3$ cleavage or a transketolase action ; with another one (26, 27) an excess of lactate was the major endproduct, which could not be easily explained by this type of cleavage. The metabolism of D-xylose is preceded by a phosphorylation which is, however, not effected by a 'xylokinase', since only a trace of D-xylose phosphate is detectable after reaction in the presence of purified enzyme preparation (17, 24). It appears more probable that D-xylose is first transformed by an adaptive enzyme, related to the xyloisomerase of *Pseudomonas hydrophila* (28) and *Lactobacillus pentosus* (21).

From quantitative estimations on the ribose-5-phosphate (R-5-P) decomposition by extracts of *A. cloacae* (24) it appears that transketolase is not the only pathway by which R-5-P is transformed into hexose. In fact, when the action of transaldolase is prevented by addition of diphosphopyridine nucleotide (DPN), cysteine and arsenate, sedoheptulose phosphate accumulates, but never more than 53 % of the theoretical amount, and hexose phosphate is still formed, thus obviously by another pathway. This is in agreement with the experiments of Horecker *et al.* (30) on liver metabolism. Cohen (4) and McNair Scott and Cohen (8) concluded that in growing *E. coli* cells, 14 % of the glucose molecules follow the HMP oxidative route, while resting cells use another (the glycolytic) pathway. Nevertheless the very active glucose-6-phosphate (G-6-P) and 6-phosphogluconate (6-PG) dehydrogenase allow 31-86 and 44 % of the glucose to be consumed by this way. The suggestions that both dehydrogenases are not the only mechanism utilized in the formation of ribose from glucose (31), that 6-PG is partly metabolized by a pathway different from the 6-PG dehydrogenase (8), that fructose-6-phosphate is metabolized by an unknown mechanism (32) and that R-5-P is transformed into hexose phosphate by a mechanism different from transaldolase and transketolase (24), need further investigations.

Since two members of this family contain the enzymes of the HMP oxidative route, we investigated the presence of this system in other genera of the same family. It appears that a very active G-6-P and 6-PG dehydrogenase

is present in all species investigated of *Escherichia*, *Aerobacter*, *Paracolobactrum*, *Serratia*, *Klebsiella* and *Salmonella*.

The oxydation of R-5-P in the presence of TPN is always preceded by a lag period, indicating the conversion of R-5-P into G-6-P. The HMP oxidative pathway will be of minor importance in the genera *Proteus* and *Erwinia*.

*The HMP oxidative route in other micro-organisms*

This pathway is also present in *Corynebacterium creatinovorans* (33). It has been reported in *Acetobacter suboxydans* (34) in *Streptomyces coelicolor* (35) and in some moulds (36).

Table I summarizes some results, available in the literature, on the presence of G-6-P and 6-PG dehydrogenase in several micro-organisms. However, we should like to warn against a too rapid interpretation of such results in favour of the presence of the HMP oxidative route in some of those micro-organisms. Several members of the family of the Bacillaceae apparently contain this system; *e. g. Bacillus subtilis* contains all the enzymes of this system, up to the stage of the sedoheptulose phosphate formation. However, R-5-P is not oxidized in the presence of TPN and the analysis of the endproducts of its decomposition shows that it is not transformed into hexoses, but into another unidentified compound, detectable by the cysteine reaction of Dische (24). Several species of *Pseudomonas*, *Xanthomonas*, *Agrobacterium*, *Rhizobium* and *Azotobacter* contain an enzym system, different from the HMP oxidative route. The lactic acid bacteria apparently contain still another type of metabolism. The discussion of both mechanisms is beyond the scope of this report.

TABLE I

*Glucose-6-phosphate (G-6-P) and 6-phosphogluconate (6-PG) dehydrogenases in micro-organisms*

| Micro-organisms | G-6-P | 6-PG |
|---|---|---|
| Algae | | |
| *Tolypothrix lanata* | TPN (37) | TPN (37) |
| *Ceramium rubrum* | TPN (37) | TPN (37) |
| *Ulva lactuca* | TPN (37) | TPN (37) |
| Moulds | | |
| *Penicillium chrysogenum* | TPN (36) | TPN (36) |
| *Neurospora crassa* | TPN (38, 39) | TPN (38) |
| *Aspergillus niger* | TPN (40) | |
| Yeasts | | |
| *Saccharomyces cerevisiae* | TPN (41, 42) | TPN (43, 42) |
| Actinomyces | | |
| *Streptomyces scabies* | TPN (35) | TPN (35) |
| *Streptomyces coelicolor* | TPN (35) | TPN (35) |
| Bacteria | | |
| *Escherichia coli* | TPN (5, 6, 8) | TPN (5, 6, 8) |
| *Aerobacter cloacae* | TPN (24) | TPN (24) |
| *Bacillus subtilis* | TPN (44, 24) | TPN (44, 24) |
| *Bacillus megatherium* | TPN (44, 24) | TPN (44, 24) |
| *Bacillus brevis* | TPN (24) | TPN (24) |
| *Corynebacterium creatinovorans* | TPN (33) | TPN (33) |
| *Pseudomonas aeruginosa* | TPN, DPN (45) | |
| *Pseudomonas fluorescens* | TPN, DPN (46) | TPN, DPN (46) |
| *Leuconostoc mesenteroides* | TPN, DPN (47) | DPN (48) |
| *Azotobacter vinelandii* | TPN, DPN (49) | |

DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide.

*2-ketogluconate-6-phosphate and the HMP oxidative route*

It has been suggested by Lipmann (50) and Dickens (51-52) that the first oxidation product of 6-PG is 2-ketogluconate-6-phosphate (2-K-6-PG). Horecker *et al.* (53) postulated the intermediate formation of a 3-ketogluconate-6-phosphate to explain the formation of D-ribulose-5-phosphate (Ru-5-P) from 6-PG. The discovery, isolation and purification of 2-K-6-PG (19) by action of an adaptive 2-ketoglucono-kinase from *Aerobacter cloacae* allowed an experimental approach of these hypotheses. This kinase is also present in several species of *Escherichia*, *Paracolobactrum*, *Serratia*, *Bacillus* and *Pseudomonas*. It is very weak in *Erwinia and Xanthomonas* (54).

In *Aerobacter cloacae* we discovered an enzyme, called 2-ketogluconate-6-phosphate reductase, catalyzing the following reaction :

$$\text{2-K-6-PG} + \text{TPNH} + \text{H}^+ \longrightarrow \text{6-PG} + \text{TPN}^+$$

6-PG could be demonstrated as endproduct of this reaction by a special paper chromatographic technique.

With DPNH this reaction proceeds at only one twentieth of the rate with TPNH. 2-ketogluconate and

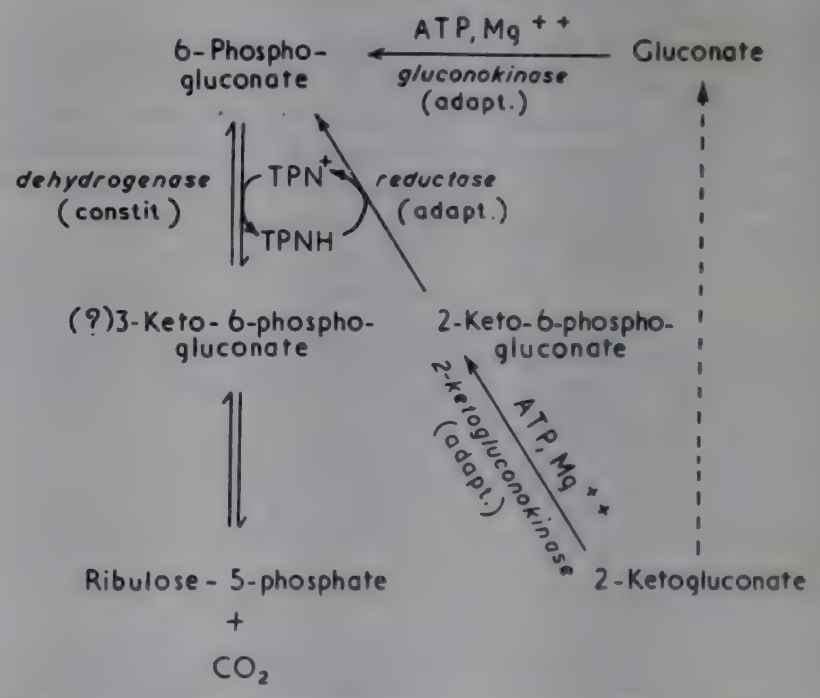


FIG. 1. — The place of 2-keto-6-phosphogluconate in the carbohydrate metabolism of *Aerobacter cloacae*.

2-keto-3-deoxygluconate-6-phosphate are also slowly reduced by this enzyme. 2-K-6-PG is not decarboxylated, neither is it cleaved by an aldolase, nor oxidized with DPN. It is oxidized with TPN, but only after a lag period indicating its previous transformation into other compounds. 2-K-6-PG reductase is not the reverse reaction of 6-PG dehydrogenase. It may be concluded from the present experiments that 2-K-6-PG

is not an intermediate in the HMP oxidative route, but only a pathway leading to it, used by some bacteria to enable them to use 2-ketogluconate as a carbon source (figure 1). The following experimental evidence corroborates this opinion :

(*i*) 2-K-6-PG reductase is an adaptive enzyme. It is about 25 times more active in adapted than in non-adapted *A. cloacae*. 6-PG dehydrogenase is a constitutive enzyme.

(*ii*) When a crude extract of adapted *A. cloacae* is fractionated with ammonium sulfate, there is no proportionality between 6-PG dehydrogenase and 2-K-6-PG reductase activity in the different fractions.

(*iii*) One of these fractions does not contain 6-PG dehydrogenase, but still contains an active 2-K-6-PG reductase.

(*iv*) 2-K-6-PG is decomposed, after a lag period, by purified preparations of adapted and non-adapted *A. cloacae* with formation of pentose, sedoheptulose and hexose. This happens only in the presence of a catalytic amount of TPN. The decomposition of 2-K-6-PG with extracts of non-adapted *A. cloacae* proceeds much more slowly.

(*v*) Extract of the following tissues possess an active or very active 6-PG dehydrogenase, although they do not reduce 2-K-6-PG in the presence of TPNH : several worms (*Dicrocoelium dendriticum*, *Ascaridia columbae*, *Toxacara canis*), barley germs, several organs of the albino rat (liver, brain, kidney spleen, skeletal muscle) and ox adrenal cortex. These rat and ox tissues have also been shown by various other methods not to metabolize 2-K-6-PG.

Baker's yeast contains a weak 2-K-6-PG reductase. The metabolism of 2-K-6-PG in these extracts follows the same pathway as in *A. cloacae*, although much more slowly. This enzyme has probably no physiological importance in yeast. Appreciable amounts of 2-K-6-PG aldolase could not be detected in yeast extract.

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# The present status of the induced synthesis of enzymes (*)

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(*Received* 15 *November* 1955)

## INTRODUCTION

For over 60 years there have existed in the microbiological literature a series of observations subsumed under the title 'enzymatic adaptation', in which a particular compound apparently evokes a well-defined change in the enzyme patterns of cells grown in its presence. The last decade has witnessed a renewed interest in these and analogous findings. Their genetic and enzymological aspects have been re-examined with the aid of more rigorous techniques and methodologies than those available to the earlier workers. These newer procedures made it possible to show in a number of cases that the phenomenon of enzymatic adaptation possessed the following two important features : (*a*) the changed enzymatic activity was not due to the selection of preexistent mutant types but rather to an induced enzymatic modification against a constant genetic background; (*b*) the observed change in enzymatic activity could be ascribed to the appearance of active apoenzyme rather than to the accumulation of cofactors or intermediates unique to the metabolism of the inducing substrate.

These researches which established these properties have been thoroughly summarized and discussed in recent reviews (1, 2, 3) and need not be detailed again here. Certain consequences (4, 5) may, however, be noted. Theoretically these findings possess obvious implications for the problem of gene function. They establish that possession by a cell of a particular gene in its nucleus does not thereby guarantee that the corresponding enzyme will be found in the cytoplasm in utilizable amounts. Thus, predictive description of phenotype from a knowledge of genotype alone is impossible even at the basic level of enzymatic constitution. It was necessary, therefore, to revise such statements as « genes control enzyme synthesis » to read « genes control the potentiality of enzyme synthesis ».

While such theoretical implications are of great interest, it was the emergent experimental consequences which proved to be of greater importance. In successfully meeting the two criteria mentioned above, an unequivocal demonstration was provided that the induced synthesis of specific enzymes could be attained under relatively simple and controllable conditions against a constant genetic background.

It was evident that the system had been found and defined which converted into experimental reality the possibility of inquiring into the mechanism of enzyme synthesis. It is the purpose of the present essay to summarize the information derived from the study from such systems.

The very nature of the phenomenon virtually dictates the kinds of questions which are initially posed. The presence of certain agents called inducers can, in the presence of a suitable energy supply, stimulate the formation of specific enzymes. The use of inducible systems to elucidate the mechanism of enzyme formation resolves itself quite naturally into attempts to provide adequate answers to the following questions :

(*a*) What is the nature of the precursor material which is transformed into active enzyme molecules ?

(*b*) What is the nature of the enzyme forming mechanism which converts the precursor material into active enzyme ?

(*c*) What is the role of the inducer, the presence of which specifically stimulates the appearance of the corresponding enzyme ?

Our knowledge of inducer function will be extensively discussed and critically analyzed in Dr. Pollock's contribution to the present symposium. We shall be primarily concerned here with the first two questions noted above and consider the inducer problem only insofar as it is directly related to the nature of the enzyme forming mechanism.

## THE NATURE OF THE PRECURSOR

The problem of the precursor is perhaps most dramatically exhibited by considering inductions carried out under the simplest circumstances. It has been shown by a number of workers with a variety of systems (1, 2, 3) that enzyme synthesis can be induced in the absence of a nitrogen source, in cells suspended in a buffer solution of the inducer. That the appearance of enzyme activity actually involves the formation of enzyme has been

(*) The original investigations described stemming from the author's laboratory were aided by grants from the National Cancer Institute of the U. S. Public Health Service and from the Office of Naval Research.

established in these cases by exhibiting the homologous enzyme in extracts prepared from the induced cells. In such inductions, the nitrogen employed by the cell in fabricating the new enzyme molecule must come from some pre-existing nitrogenous compounds and one is immediately faced with the obvious necessity of identifying the components so employed.

Before considering the most recent experiments which have led to a satisfactory resolution of this problem, it is of interest to note briefly some of the earlier work which, although inconclusive, nevertheless exerted a strong influence on the subsequent development of this aspect of the problem. Monod (6) in his classic investigation into the growth of bacteria, discovered the existence of a severe interaction between enzyme forming systems which was expressed by the fact that simultaneous synthesis of two metabolically unrelated enzymes did not occur on exposing cells to a mixture of the two relevant inducers. In general, only one of the enzyme was formed at a time. A similar situation was uncovered in the yeasts by Spiegelman and Dunn (7). With yeast the presence of an external nitrogen supply greatly suppressed the severity and the extent of this interaction and indeed under certain circumstances simultaneous formation of two otherwise interfering enzymes were made possible.

Although these interactions were discovered relatively early in the renewed investigation of the phenomenon of enzymatic adaptation, their detailed significance remains to be delineated. Nevertheless, at the time they were interpreted to suggest a competition for some commonly required nitrogenous material and therefore implied the existence of precursor not as yet specified as to its ultimate enzymatic function. To these findings may be added those which established that the induction of enzyme requires the participation of a functional and utilizable energy generating mechanism. Agents such as 2, 4-dinitrophenol (8), sodium azide (9), and arsenate (10) which prevent the utilization of energy generated by metabolism also inhibit the induction of enzyme activity.

The importance of these early observations derived essentially from the fact that they made unlikely one quite obvious and attractive possibility of explaining the phenomenon of induced enzyme formation. This hypothesis suggests that the mechanism involved is one akin to the activation of trypsinogen to trypsin. Of course, the fact that energy is required for the induction precludes at once any simple application of this concept. In addition, such a model would suppose the pre-existence in a cell of inactive forms of enzymes already fully determined as to their specificity and their function. The inhibitory interactions observed would be difficult to explain under these circumstances. While observations of this nature could not definitely decide the issue, they clearly encouraged the search for a nitrogenous precursor not as yet restricted in its specificity and potentially convertible into more than one kind of enzyme molecule.

In designing experiments which seek to reveal the nature of this precursor, one can be guided by the fact that, in principle, the following three mechanisms of enzyme synthesis can be written down :

$$\text{complex precursor} \rightarrow \text{active enzyme} \qquad (1)$$

$$\text{complex precursor} + \text{free amino acids} \rightarrow \text{active enzyme} \qquad (2)$$

$$\text{free amino acids} \rightarrow \text{enzyme} \qquad (3)$$

Reaction (*1*) assumes the pre-existence in the non-induced cell of a complex precursor which can be converted into active enzyme without the involvement of the free amino acids. This property distinguishes it from mechanisms (*2*) and (*3*) and permits an experimental decision.

Evidently what we are asking is whether it is possible for the precursor to become active enzyme without the participation of the free amino acid pool. Putting the question this way suggests immediately the necessity for examining the effect on the synthesis of enzyme of any experimental condition which decreases the availability of the free amino acids. Several methods are available and have been employed for achieving a restriction of this nature and they may be listed as follows : (*a*) the use of amino acid analogues as specific agents to prevent the incorporation of the free amino acids into protein; (*b*) in the case of those cells which possess an internal amino acid pool, to examine the effect of depletion and replenishment of this pool under conditions which would minimally disturb other components of the cell; (*c*) the use of amino acid deficient mutants which would make unavailable specific components.

Experiments along all of these lines have been realized with yeast and the bacteria. The following paragraphs summarize briefly the evidence obtained :

*The effect of amino acid analogues on enzyme synthesis*

Halvorson and Spiegelman (11) carried out a study with a series of more than 40 analogues of amino acids for their effects on induced formation of α-glucosidase in *Saccharomyces cerevisae*. A parallelism was established between the capacity of an analogue to inhibit net protein synthesis, as measured by growth, and its ability to suppress enzyme synthesis. In the case of the effective analogues, complete and specific reversal of the inhibition was achieved by the addition of the homologous amino acid. The generality of these findings was extended by the independently performed experiments of Lee and Williams (12) who demonstrated that the administration of ethionine to the intact rat prevented the formation of tryptophane peroxidase. In the experiments with the yeast (11, 12, 13, 14), it was possible to demonstrate by direct analysis that the presence of an effective amino acid analogue inhibits incorporation from the free amino acid pool into the protein fraction. One interesting feature which emerged from these experiments is that the presence of any one of the active amino acid analogues prevents the incorporation not only of its homologue but also virtually of all the other amino acids as well. Further, no peptide fragments, unique to pools derived from cells incubated with an amino acid analogue, could be found.

In these studies, no evidence for an amino-acid-independent transformation of a complex precursor into active enzyme was obtained. The data rather led to the conclusion that the primary pathway of the induced formation of enzyme in non-dividing cells of yeast involves the compulsory utilization of the internal free

amino acids. The fact that the utilization of non-homologous amino acids was blocked concurrently suggested further that the first stable intermediate formed in the synthesis of an enzyme molecule is of such a complexity as to demand the simultaneous availability of a large portion of the component amino acids.

### *The effect of the availability of free amino acids on enzyme synthesis*

If the conclusions derived from the experiments with amino acid analogues are correct, it would be expected that the ability of cells to form enzyme should parallel the availability of free amino acids for protein synthesis.

One striking difference between the enzyme forming capacity of yeast as distinguished from that of many gram negative bacteria receives simple explanation in these terms. Thus, yeasts are able to form enzymes when suspended in nitrogen-free solutions of inducer, whereas, the gram negative bacteria in general require an exogenous supply of nitrogen as a necessary concomitant of enzyme synthesis. The work of Taylor (15) suggests a reasonable explanation for this apparent independence of the yeast enzyme synthesizing mechanism. This investigator surveyed a variety of yeast and bacteria for the presence of free amino acids in their internal environment. Of the three yeast types examined, all possessed detectable quantities of the five amino acids looked for. Amongst the bacteria, the gram-positives possess primarily glutamic acid and lysine. None of the gram-negatives included in the survey contained detectable free amino acid by the procedures employed.

The possession of an internal pool was subsequently found to be a universal attribute of a wide variety of yeasts (16). It would appear that the ability of yeast to get along without an external source of nitrogen for enzyme synthesis is due to the fact that they have internal supply. To examine the question then of the effect of free amino acid availability on enzyme formation in the yeast it was necessary to devise and employ procedures capable of modifying these pool levels both quantitatively and qualitatively. Using such procedures, Halvorson and Spiegelman (17) demonstrated a strong correlation between enzyme forming capacity and pool level in both depletion and replenishment cycles. The results obtained in these studies supported the conclusion that free amino acids constitute the quantitatively predominant source of nitrogen in the formation of new enzyme molecules. Again, no evidence was uncovered which suggested the existence of an amino-acid-independent transformation of a pre-existing complex precursor into active enzyme molecules.

### *Enzyme formation by amino acid auxotrophic mutants*

The third approach mentioned which could provide relevant information involves the use of the auxotrophic mutants deficient in the ability to synthesize one or another of the amino acids. It was obvious that organisms such as yeast, which accumulate an internal free pool would be difficult to employ in such studies and the not unexpected difficulties arose when they were attempted with the yeasts. Fortunately, however, this approach was successfully applied almost simultaneously in two laboratories and it is interesting to not that in both instances the organisms employed, *Escherichia coli* and *Aerobacter aerogenes*, possess a vanishingly small internal supply of free amino acids.

One of these investigations stems from the illuminating studies of Monod and Cohn (1) and their collaborators into the formation of β-galactosidase by the ML strain of *E. coli*. It is interesting to note that in the course of these studies, Cohn and Torriani (18, 19) had discovered the existence of an enzymatically inactive protein (Pz) which was serologically related to the β-galactosidase. In addition to this obvious structural relationship, they established a suggestive correlation between the distribution of the Pz protein and the capacity of the cells to synthesize the β-galactosidase. Finally, they also showed that a significant decrease in Pz occurred in cells during the induced synthesis of β-galactosidase. Although not the only possible hypothesis entertained by the authors, it is clear that all of these observations would receive ready explanation if Pz were indeed the precursor of the β-galactosidase. In any event, taken together the observations noted offered the most impressive evidence existent in the literature to support the suggestion that a pre-existent complex specific precursor is involved in the synthesis of a known enzyme.

Monod, Pappenheimer and Cohen-Bazire (20) undertook to investigate this question further by employing a series of mutants, each of which was deficient in the ability to synthesize a single amino acid. These mutants were subjected to a 'specific starvation' by being grown in a medium in which the required amino acid was present in limiting quantities, whereas all other compounds were in excess. Immediately upon the cessation of growth which attended the exhaustion of the amino acid, an inducer of the β-galactosidase was introduced. It was found that little or no enzyme was synthesized by cells so treated, despite the fact that they contained normal amounts of Pz. Such cells do, however, form enzyme immediately upon the addition of the amino acid they are unable to synthesize. These results made it necessary to abandon any interpretation of the relation between Pz and the β-galactosidase which involves a direct, amino-acid-independent conversion of Pz into active enzyme. The large number of amino acid auxotrophs employed in this study would suggest further that if Pz is indeed a precursor, a considerable number and variety of amino acids must be added to it before it is converted into active enzyme.

Ushiba and Magasanik (21) employed essentially the same approach in their study of the adaptive utilization of myoinositol by mutants of *A. aerogenes*. The results obtained led these authors also to the conclusion that the induced formation of the enzymes they were studying involved extensive synthesis from the amino acids. In a subsequent study, Rickenberg *et al.* (22) reported similar experiments and results with *E. coli* strain K12.

### *Is there a pre-existent complex precursor ?*

The experiments cited thus far make unlikely any mechanism of synthesis which presumes the conversion of a pre-existent precursor into enzyme by a process which is independent of the free amino acids. The only alternatives left are, either that there is no pre-existent

complex precursor, or that one does exist but becomes active enzyme only after the incorporation of amino acids. The most obvious experimental approach aimed at a choice between these alternatives would appear to be the use of isotopic labels. Thus, the induction of enzyme synthesis in uniformly labeled cells suspended in unlabeled medium should provide the necessary data providing the enzyme synthesized can be isolated in a pure state and its isotopic content determined. Rotman and Spiegelman (23), and Hogness, Cohn and Monod (24) independently undertook to provide data relevant to this issue, using the β-galactosidase system in *E. coli*.

Rotman and Spiegelman (23) secured uniformly labeled cells by growth in $^{14}C$ lactate. Enzyme was induced for short periods while the cells were suspended in non-radioactive medium. The β-galactosidase synthesized was isolated and purified by means of zone ionophoresis through starch columns. Further purification was achieved with the aid of specific precipitation with purified antiserum. The results obtained revealed that less than one percent carbon of the newly formed enzyme molecules could have been derived from any cellular components existing prior to the moment of the addition of the inducer. In the experiments of Cohn, Hogness and Monod, $^{35}S$ was employed as the isotopic label and similar methods for the isolation of the enzyme. Identical results and conclusions were obtained.

These findings virtually eliminate any hypothesis which assumes the pre-existence of a complex precursor material which is convertible into enzyme. It is evident that a mechanism suggesting the *de novo* formation of enzyme from amino acids is at present the only one which has received experimental support.

A consequence of considerable importance issuing from this last conclusion is that induced enzyme synthesis is thereby equated to the process of protein synthesis. It follows that data derived from the study of enzyme induction are pertinent and relevant to the more general problem of protein formation. Further, the use of inducible enzymes as model systems of protein formation can, in principle and in fact, confer two significant operative advantages. In the first place, one is assured that the synthesis of a protein is being examined, a certainty not available to experiments dependent solely on incorporation studies. Secondly, the formation of as little as 0.01 μg. of new protein can be detected with ease and precision.

## PRECURSOR AND THE NATURE OF THE ENZYME FORMING SYSTEM

In the present discussion, the term 'enzyme forming system' hereinafter referred to as EFS, will be used to designate that structure in the cell which is directly and personally involved in the process of fabricating the enzyme molecule. This verbal device is employed to isolate the EFS conceptually from all the other cellular components which can and probably do intervene more or less directly in the synthetic process. It will, of course, be noted that there is an assumption made here. By so stating the problem we do presume the existence of such a unique structure and, at least implicitly, ignore the possibility that proteins and enzymes are formed by a multitude of cooperating and sequential reactions.

In thinking about the possible nature of the EFS and in designing experiments to clarify the mechanism of its functioning, it is difficult to avoid being influenced by the results of the investigations into the precursor question. In a sense, these findings force the institution of an active search for EFS. The data we have reviewed relevant to the precursor problem are satisfyingly clear-cut, almost distressingly so. They lead compellingly to the conclusion that in fabricating a new enzyme molecule, the cell prefers to weave it rather than to stamp it into existence. In this process, the simplest components are employed. Further, we find no evidence for any stable intermediates smaller than that requiring the presence and utilization of all the amino acids. A step wise formation beginning with simple peptides and proceeding through polypeptides of intermediate lengths would appear to be eliminated. From one point of view, this is of course a pessimistic conclusion. It suggests that a successive approximation to an understanding of how proteins are synthesized will not be achieved in terms of a gradually better insight gleaned from the study of intermediate pieces of increasing complexity as they approach the final stage of synthesis. A door is thus slammed upon an extremely attractive approach for tackling the question of protein formation. Those who accept these conclusions obviously are faced with the necessity of finding a new approach to the solution of the problem of protein synthesis.

Adopting a mechanism of protein synthesis which involves the simultaneous availability of the constituent amino acids leads one quite naturally to consider a template type mechanism. It is unnecessary here to undertake an extensive discussion of the recent information (25, 26, 27, 28) obtained from radioactive experiments with intact animals and tissues which have attempted to decide between template and step wise mechanisms. In general, unequal labeling of the protein has been taken as an argument against the template hypothesis. That this is not a necessary deduction from such data has already been pointed out by Dalgliesch (29).

A more serious difficulty is introduced by the realization that the incorporation of labeled compounds need not provide us with information relevant solely to the question of total protein synthesis. Exchange reactions are necessarily included in the information obtained from such experiments. That a very real difficulty exists is well illustrated by the recent experiments reported by Gale and Folkes (30, 31) in which a dissociation of net protein synthesis from incorporation by exchange reactions has been demonstrated. As had been shown with yeast (11), it was found that *p*-chlorophenylalanine can prevent new protein synthesis in *Staphilococcus aureus*. In addition, Gale and Folkes discovered that although the presence of this analogue effectively prevents the incorporation of phenylalanine into the protein, it has relatively little effect on the exchange incorporation of glutamic acid.

The existence of such phenomena makes it difficult to interpret with certainty, the significance and uniqueness of data derived solely from incorporation studies.

## THE RELATION BETWEEN TEMPLATE ENZYME AND 'EFS'

The data reviewed thus far would indicate that enzyme molecules are fabricated on a template. The investigations by Pollock (32, 33, 34) of the penicillinase synthesizing system of *Bacillus cereus* suggest that inducer molecules appear to be specifically bound to some site concerned with enzyme synthesis. Identification of the inducer complexing site with the template is the simplest and most economical hypothesis at present worthy of further exploration. The question we should like to consider for the moment is whether this is all there is to the EFS. Essentially what we are asking may be put as follows : Does the combination between the inducer and empty template occur readily, and if so, does this then lead immediately to full function ?

The weight of the accumulated evidence would appear to answer the question posed in the negative with respect to both points. The data suggest that inducer combines relatively poorly with unoccupied template and that this combination *per se* does not lead to full function. Inasmuch as the relevant data have been extensively discussed elsewhere (35), we may forego here a detailed repetition and confine ourselves to an enumeration of the key findings and a brief description of the resultant picture which emerges for the components of the EFS.

In the first place, the kinetics of enzyme formation argue against such a simple activation hypothesis for template function. An uncomplicated application of this mechanism would suggest that, providing other conditions are not limiting, enzyme synthesis should proceed at its maximal rate immediately upon the adsorption of inducer. Actually, a careful examination of the early phases of induction reveals the presence of a rising rate of enzyme formation suggesting the occurrence of a self-reinforcing activation of the EFS in the early stages of induction. It should be noted that this is observed (36) even under conditions of 'gratuity' where the enzyme formed does not participate in the generation of energy for biosynthesis.

The interesting discovery of 'long-term adaptation' by Winge and Roberts (37) made it possible to examine the autocatalytic nature of induction by genetic means. In the course of investigating the nature of long-term adaptation, a tool of surprising subtlety and power was evolved. Spiegelman, Sussmann, and Pinska (38) showed that in strains exhibiting this new and pathological type of induction the vast majority of the cells were in fact not inducible. The small number of positive cells, however, passed on their enzyme forming ability indefinitely to their progeny so long as growth occurred in the presence of inducer. However, such positives reverted to the negative state quite abruptly after five or more divisions in the absence of inducer. This mass change in phenotype virtually eliminated any mutational interpretation of the reversion phenomenon or of the origin of the positives themselves. The autocatalytic property of the EFS, or some necessary portion of it, is here dramatically exhibited in terms of the transmission of enzyme synthesizing capacity from mother to daughter cell. Some functional EFS is apparently needed to make more. If by chance a cell is produced with none, it and its progeny are negative.

A detailed quantitative analysis of the reversion to the negative state of both mass populations and single cell pedigrees led to a relatively precise theoretical description of the reversion process (38, 39). The loss of enzyme forming ability followed exactly the pattern to be expected from the dilution of particulate elements capable of autocatalytic increase in the presence of inducer and necessary for enzyme formation. The detailed data could be accurately described in terms of the quantitative consequences of the following set of statements : (*a*) the distribution of active particles among cells is assumed to be normal; (*b*) during growth in an inducer-free medium, these particles neither decrease nor increase in absolute number, and hence the average number per cell decreases exponentially; (*c*) the active particles are distributed at each division randomly with equal probability between mother and daughter cells; (*d*) the minimal number of active particles that a cell must contain in order to score as a positive is close to one.

These properties were derived solely from a study of the transformation from the positive to the negative state. Analysis of the reverse process quickly led to other features which illuminated further details of the system. Rotman and Spiegelman (40) were able to convert a large percentage of negatives to the positive phenotype by treatment with fractions of yeast extract. It was possible to show that each of the positive cells so obtained acquired only a few active particles as a result of the conversion. On the basis of the properties and origin of preparations possessing converting activity it was proposed that the transformation of a negative into a positive cell does not involve the *de novo* formation of active particles but rather the activation of preexistent inactive units. We thus, then, have two categories of particles, active and inactive.

A third type was revealed in an analysis by Campbell and Spiegelman (35) of the growth of active particles. This third kind is characterized by being easily converted by inducer alone to an active unit, an event which is rare with the inactive particles. We designate this intermediate type as a 'convertible' particles. Their presence is indicated by an abrupt rise in active particle number when reverting positive cells are exposed to inducer. Subsequent increase of active particles then obeys an exponential law. The convertible particles occur with increasing frequency as positive cells are allowed to go through dilution growth in the absence of inducer. Further, they appear to suffer decay since if they are not brought into contact with an inducer soon after their appearance, activation by an inducer is no longer possible.

Another peculiar feature which emerged from the late portion of the reversion is an anomalous change in stability of active particles. It was shown in one of the earlier studies noted (38) that active particles are perfectly stable in the early reversion divisions. However it was found (41) that this stability disappeared after about the seventh division in the absence of inducer.

## THE FEED-BACK MODEL OF THE 'EFS' RELATING TEMPLATE, ENZYME, AND INDUCER

We should now like to essay a synthesis of the biochemical, kinetic, and genetic information thus far summarized in terms of the simplest model consistent with the observations.

The model to be described is essentially the one designed by Campbell and Spiegelman (35) in an attempt to explain certain paradoxical aspects of the growth kinetics of active particles in long-term adapting strains. Its major and unique features are most easily seen and devoloped in terms of the properties of such strains. It will, however, be evident that the scheme is perfectly general and applicable to normal enzyme synthesis.

The biochemical investigations of the precursor aspect of enzyme formation led to the conclusion that a template is involved. The kinetics of normal induction and the genetic data obtained with the aid of the slow strains both suggest that induction is characterized in its early stages by an autocatalytic activation. All these rather diverse data receive, therefore, simple explanation by the assumption that initially the templates are relatively inactive and are autocatalytically converted to full activity during the course of induction. A unified description is thus possible if we permit ourselves to identify the autocatalytic active particles defined by the genetic operations with the autocatalytically activated template suggested by the biochemical studies.

We now inquire where in our model the inducer is likely to fit and how it is tó function. Both the kinetic and genetic experiments indicate that active templates increase in number during exposure to inducer. Removal of the inducer, on the other hand, results in a complete cessation of the increase but the number present at the moment of removal remains constant for many hours. The simplest explanation which can be offered to explain this fact is that the inducer or some derivative of it is irreversibly incorporated into the structure of the active template. If one proceeds along this line of reasoning, one difference between an active template and an inactive one would be that only the former contains inducer. However, if this were the only difference, exposure to inducer should convert negative slow cells into positive ones because in one generation half of the templates would have been formed in the presence of inducer. This suggests, then, that an active template differs from the inactive form in some property other than the possession of the inducer. The possibility is, therefore, provided for a third template type, neither active nor inactive, possessing the second property but not the inducer. A description is thus provided for the convertible template which becomes stabilized in the active form on the addition of inducer.

We now turn to a consideration of the likely nature of this 'second property' possessed in common by active and convertible templates but lacking in the inactive variety. For simplicity and ease in following the argument, we may here summarize the properties which any model constructed on the basis of the above discussion must exhibit.

(*i*) Each cell contains a certain number of templates specific for the synthesis of some enzyme. They may exist in the active, inactive, or convertible forms.

(*ii*) In the presence of inducer, a cell containing one or more active or convertible templates will give rise within a few hours to progeny in which most or all of the templates are active.

(*iii*) A cell containing no active or convertible templates may grow for many generations in the presence of inducer without any templates ever becoming active. If activation does occur, it is a rare event. It is, however, heritable on the cellular level when it does occur as required by property (*ii*).

(*iv*) Active templates differ from the convertible ones in that the former contain inducer. Convertible templates are readily transformed to active ones by the addition of inducer.

(*v*) Active templates form the enzyme mentioned under (*i*); inactive ones do not.

From properties (*ii*) and (*iii*) it is clear that the presence of some active templates in the cell greatly accelerates the action of others. Whether this includes other templates already present or only those subsequently formed is not deducible from the data. The question is, however, how this activation might take place.

The two simplest possibilities are : (*a*) The active templates self-duplicate ; the new ones being, so to speak, decended from the one originally present ; (*b*) active templates produce something which activates inactive templates.

Both may operate ; however the functioning of the second mechanism is already directly implied by the experiments of Rotman and Spiegelman (40) which show that inactive sites pre-exist and can be converted to active ones. Under the circumstances, the self-duplicating property can be abandoned as being superfluous for a description of enzyme forming system as a protein synthesizing machine. What the active templates might produce which would activate other ones is also not deducible from the existent information. However, the only thing the template can be presumed to produce heterocatalytically is enzyme. One is then led from this model to conjecture that the enzyme molecule itself is the activator of inactive templates. The picture of the mechanism emerging is clear and we may now designate templates by T, inducer molecules by I, and enzyme by E. We postulate then the following :

(*i*) When cells have been grown for many generations in the absence of inducer, their templates are all, or nearly all, in the simple and inactive form T.

(*ii*) The complex T-E is unstable and can occur in either one of two ways. In a non-induced cell each T has a small probability of fabricating spontaneously an enzyme molecule. Secondly, an induced cell allowed to grow in the absence of inducer will, when the inducer is sufficiently diluted, produce T-E from decomposition of the inducer-T-E complex mentioned in the next statement.

(*iii*) The reaction :

$$\text{T-E} + n\,\text{I} \rightarrow \text{T-I}_n\text{-E}$$

is rapid and relatively irreversible. Here, $n$ is the number of inducer molecules bound per T-E complex. $n$ may be unity in some cases and greater in others.

(*iv*) A population of cells grown in an inducer containing medium has most or all of its sites in the form of $\text{T-I}_n\text{-E}$.

(*v*) Of the forms mentioned above, only $\text{T-I}_n\text{-E}$ can effectively and rapidly catalyze enzyme synthesis.

In the nomenclature of Cohn and Monod (44), T would be an apo-organizer; I, a co-organizer; and, T-I, an organizer. The complexes T-E and T-$I_n$-E represent new entities not embraced or employed by their terminology.

The reversion from positive to negative in 'slow adapters' can be interpreted in terms of the above model in the following manner. As soon as growth in a galactose-free medium occurs, all newly formed templates appear as either T or T-E and consequently no enzyme synthesis takes place in them. The templates which were present initially remain as T-$I_n$-E and continue to synthesize enzyme. They are the autocatalysts or active particles and are diluted out by growth. As the reversion growth proceeds, one gets, as a result of the consequent dilution of inducer, some convertible particles, T-E. The ultimate and irreversible decay of active particles if the reversion proceeds too long receives, therefore, simple explanation in terms of the instability of T-E. The anomalous change in stability of active particles which occurs late in the reversion is explained simultaneously.

The conversion to the positive state occurs when a cell containing one or more templates of the form T-$I_n$-E or T-E is placed in a medium containing inducer. Any T-E complexes are first converted to T-$I_n$-E, and then in both cases free enzyme is formed. By virtue of the enzyme produced other templates can be rapidly converted to the stable T-$I_n$-E state, provided excess inducer molecules are present. The infrequent spontaneous production of positives from negatives in the slow strains might be explained on the basis of the rare occurrence of enzyme formation by unoccupied T or T-$I_n$.

The novel function attributed to the enzyme and inducer molecules recalls the formally similar hypothesis of the plasmagene theory. It should be emphasized, however, that the present model exploits a feed-back feature which was actually inherent in the plasmagene model but not used. It is the recognition that enzyme molecules can serve to activate unoccupied templates that permits an explanation of autocatalysis which does not invoke self-duplication. There are no data existent to our knowledge which demand this latter property as an integral part of the enzyme forming process. It would, however, seem necessary to retain self-duplication as a method of template maintenance to explain the instances of cytoplasmic transmission noted, particularly in the case of the *petits* mutants.

Our discussion of the biochemical, the kinetic, and the genetic information available on enzyme induction has led us to postulate three components of the enzyme forming system, and they are inducer protein, and template. In any given case, the first two can be reasonably well defined in terms of known chemical entities. We now turn our attention to the question of the identification of the chemical nature of the third member of the triad, the template.

## THE CHEMICAL NATURE OF THE TEMPLATE

A template which is to serve as a device for protein synthesis must be at least as complicated and as large as the molecule which it is forming. Other than the protein molecule itself, there are relatively few candidates one can propose which can satisfy the two criteria of size and informational complexity. With these restrictions in mind, the two known possibilities are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

In the following paragraphs we will briefly examine the available evidence for and against the possibility that one or the other of these substances is associated with the enzyme forming mechanism.

### *DNA as a component of (EFS)*

Evidence from work with the transformation principles offer convincing evidence that genetic information can be stored in and transmitted through DNA. The potentially, therefore, of forming any specific kind of protein molecule must ultimately be referable to the DNA of the cell. The question, however, which we should like to entertain at present is whether DNA is directly and personally involved in the synthesis of protein or whether it effects its influence *via* an intermediary. Definitive evidence one way or the other is, at present, not available. Presumably, an unequivocal demonstration will ultimately emerge from experiments analogous to those being performed in the laboratories of Brachet (45) and of Mazia (46, 47) with enucleated fragments of amoeba. At present, the best that can be offered is a series of experiments inquiring whether a correlation can be established between the metabolic activity or state of DNA and the act of protein synthesis.

There exists a variety of experiments in which it is possible to demonstrate a complete dissociation of DNA metabolism from protein synthesis. DNA formation is known (43) to be far more sensitive to inhibition by radiation with X-rays than is protein formation. Baron, Spiegelman and Quastler (48) have shown that X-ray dosages, far exceeding those expected to stop the formation of DNA completely, permit normal enzyme formation in yeast. Similar dissociations have been achieved with other systems and by different means (49, 50).

Kelner's (51) studies on photoreactivation of *E. coli* following exposure to ultraviolet have provided an elegant method for a virtually complete separation of RNA and protein formation from net DNA synthesis. Halvorson and Jackson (52) employing yeast have recently repeated and confirmed these results. The results obtained suggest again that protein and RNA continue to be synthesized subsequent to UV doses which completely inhibit the DNA formation.

Cohen and Barner (53) have reported the ability of a thymineless mutant of *E. coli* which can synthesize xylose isomerase in the absence of an added supply of thymine. This important finding was confirmed in our laboratory (16) using the same mutant and examining β-galactosidase forming capacity. It was found that cells of this strain synthesized considerable amounts of enzyme when suspended in a synthetic medium lacking thymine. This behavior is in striking contrast to that observed with mutants possessing other metabolic deficiencies. Thus, in our own experience and in that of others (16, 20, 50) adenineless, uracilless or amino-acid deficient mutants form little or no enzyme in the absence of the required metabolite.

An interesting apparent exception to the information cited is that of Allfrey's (42) observation with isolated nuclei. He found that treatment with DNAse suppressed the ability of his preparations to incorporate labeled amino acids whereas RNAse had little or no effect. The situation observed here may, however, be a reflection of the low nuclear RNA content. If, as seems likely, RNA is derived from DNA, destruction of the latter would eliminate protein synthesis in such systems.

The data cited above demonstrate that drastic interference with DNA synthesis is often not accompanied by very striking effects on the formation of protein. While such findings cannot eliminate DNA as an active component of the EFS, they hardly lend support to the supposition that it is. The credence assignable to such negative conclusions with respect to DNA gains further weight from similar experiments examining RNA metabolism which yielded strikingly different results.

### *RNA as a component of EFS*

Many have postulated RNA as a key substance in protein synthesis. Chantrenne (54) has succinctly summarized such speculations and the supporting evidence. Here we would like to confine our attention to the information derived from the study of enzyme synthesis. Again, as in the case of DNA, correlative experiments have been performed with intact cells examining the effects on enzyme formation of agents or conditions which influence RNA metabolism.

*Experiments with ultraviolet light.* — Swenson and Giese (55, 56) demonstrated that exposure to ultraviolet dosages far exceeding those required to stop DNA formation, results in the inhibition of induced enzyme synthesis in yeast. Examination of the action spectrum of the inhibition revealed that it coincided with the absorption spectrum of nucleic acid. Halvorson and Jackson (52) extended these interesting observations. They examined the effects of various dosages on the synthesis of α-glucosidase, the ability to use free amino acid pool components and incorporation $^{32}P$ into the nucleotides of RNA. Their results established an excellent parallelism between the loss in capacity to utilize the free amino acids and the ability to synthesize enzyme. It was further found (16) that even slight damage of RNA metabolism, as measured by ability to incorporate $^{32}P$ had profound effects on enzyme forming ability. Thus, at a dose which achieved a 22 % inhibition of RNA metabolism enzyme formation was suppressed to the extent of 95 %.

*The effect of a uridine analogue on enzyme synthesis.* — One obvious approach which could in principle yield information pertinent to the role of RNA is to examine the effects of various analogues of uracil and its derivatives on enzyme formation. Ben-Ishai and Spiegelman (57) undertook such a study. One of the most effective compounds found was 5-OH-uridine which the experiments of Roberts and Visser (58) suggest is able to prevent the utilization of uracil for the synthesis of RNA. The presence of as little as 5 μg./ml. of this compound results in virtual cessation of β-galactosidase formation by *E. coli*. Further, this inhibition can be achieved even if the OH-uridine is introduced subsequent to the addition of inducer, at a time when maximal rate of enzyme formation had been attained.

Several illuminating facts emerged from those experiments. One was that the OH-uridine could effect a complete inhibition of β-galactosidase formation at concentrations which had no effect on over-all protein synthesis. In view of the suggested sensitivity of the β-galactosidase forming system to the supply level of the RNA precursors, the addition of amino acids might be expected to result in an inhibition of β-galactosidase formation. This prediction is experimentally realized (49). Thus, the presence of inducer fails to stimulate enzyme formation if amino acids are added simultaneously. The suppression is virtually complete for a period of a half hour following which some recovery of enzyme forming capacity occurs. That the inhibition is related to RNA precursor supply is supported by the ability of purine and pyrimidine bases to reverse it.

This dependence of enzyme formation on an adequate supply of nucleic acid precursors has also been exhibited (57) in the case of α-glucosidase formation in *S. cerevisiae*. In addition to their free amino acid pool, yeast also possess a considerable internal supply of nucleotides and their polyphosphate derivatives (59). It was found possible (57) to specifically deplete the nucleotide pool by incubation in the presence of an external supply of amino acids and energy. This treatment leads to a loss of enzyme forming capacity while leaving the free amino acid pool intact. If cells are first partially induced and their nucleotide pool then depleted, they fail to form enzyme on being again exposed to inducer. If their nucleotide pool is, however, replenished, enzyme synthesis proceeds normally. These experiments illustrate in a different manner and with another system the apparent requirement that RNA synthesis be possible if enzyme formation is to continue.

### *Experiments with subcellular fractions*

The experiments thus far described strongly implicate RNA as the template in the process of enzyme synthesis. They cannot, however, be taken as conclusive. It is painfully obvious that while interesting and perhaps even ingenious experiments can be performed with intact cells, the distance between the data and the conclusions derived from them is too great for certainty. Definitive identification of the chemical nature and the mode of action of the template is not likely until the latter has been physically isolated in a functional state. *In vitro* performance of its function by the isolated enzyme forming system may be suggesting the impossible since it demands even more than that which has already been accomplished in the case of transformation in the bacteria. In the latter, genetically competent material has been separated from other cell components. However, the transforming principles have been asked to function only after reinsertion into an intact living organism.

Nevertheless that the ideal *in vitro* situation may be attainable in the not too distant future is prophetically foreshadowed by the striking successes which have recently been recorded with subcellular fractions. Many of these deal primarily with incorporation studies. To this extent it is uncertain that they necessarily

represent model systems which will permit the further dissection of the protein synthesizing mechanism. While the data must therefore be interpreted with caution, their uniqueness and potential value commands consideration.

Zamecnik and Keller (60) succeeded in preparing a microsome fraction which actively incorporates amino acids when supplemented with some component of the supernate and an ATP-generating system. Subsequent work on the supernate fraction by Keller and Zamecnik (61) indicated the presence of an enzyme which generated guanosine-tri-phosphate a derivative of which functions in the insertion of the amino acids into peptide linkage. The work of Hoagland (62) and DeMoss and Novelli (63) strongly suggests that polyphosphate derivatives of nucleotides activate amino acids prior to their incorporation.

Lester (64) and Beljanski (65) examined the ability of lysozyme treated preparations of *Bacillus megaterium* to incorporate labeled amino acids. Both authors found that treatment with RNAse abolished this ability whereas exposure to DNAse was stimulatory.

The most extensive investigation on the properties of subcellular fractions has come from Gale's (66, 67) laboratory. In these studies, cells of *Staph. aureus* are disrupted by sonic disintegration and a fraction obtained by differential centrifugation which is relatively low in viable cells, and, therefore, presumably in intact cells. Although it is unlikely that this preparation is homogeneous, it nevertheless is of the greatest interest since it is amenable to enzymatic and extractive resolution. Removal of the nucleic acid from such disrupted cell preparations leads to a marked lowering in their ability to incorporate amino acids. This loss can be restored by the addition of nucleic acids from the same species, DNA being more active than RNA on a dry weight basis. This latter finding may be merely a consequence of the greater stability of DNA to isolation procedures. The data are consistent with the concept that the RNA made from the DNA supplied is the active agent.

A most interesting recent development has been the discovery by Gale and Folkes (68) that the presence of specific di- and tri-nucleotides are extremely active in promoting the incorporation of specific amino acids. Thus, for example, di-nucleotides containing adenine and cytosine can completely replace the intact RNA in promoting the incorporation of aspartic acid. Indeed, on an equivalent weight basis the di-nucleotide is more than a hundred times as active as the intact RNA. Interpretation of these findings is yet uncertain. It may indeed be, as suggested by Gale and Folkes (68), that these small fragments represent that part of the RNA template which is concerned with the insertion of the corresponding amino acid into peptide linkage. An argument which can be raised against this assertion stems precisely from the observed high activity of the di-nucleotides. It seems unlikely that nucleotide pairs are sufficient to specify the relevant amino acids since only 16 possibilities are uniquely determined. At least three bases of the RNA template would have to be involved in the specification of a given amino acid if 20 or more choices have to be made. This reasoning assumes, of course, that the four bases are the only components of the code.

An alternative explanation of these findings can be offered. It may be that the active fragments of Gale-Folkes may, by transfer reactions, generate the nucleotide components functioning in the activating mechanism suggested by the work of Hoagland (62) and of DeMoss and Novelli (63). Whatever the interpretation, it nevertheless remains true that these results are pregnant with many possibilities.

As distinguished from incorporation studies, the attainment of protein synthesis has been reported in only two sorts of subcellular fraction. One is the system of Gale and Folkes (67) in which the development of 'glucozymase', catalase, and the inductive formation of β-galactosidase have been demonstrated. When the preparations are sufficiently resolved by removal of RNA and DNA, it is found that RNA stimulates the formation of catalase whereas DNA is required for the formation of β-galactosidase.

Again, the relative high activity of the DNA may be a consequence of greater stability to extractive procedures. No limits to the potentialities of this system are apparent. It is difficult to believe that its future study can fail to provide definitive answers to the basic problems of template function and specificity.

Another system which gives great promise of future fruitfulness are the so-called protoplasts of *B. megaterium*. Weibull (69) showed that these could be prepared by treatment of cells with lysozyme in hypertonic medium. Wiame *et al.* (70) showed that these preparations were able to synthesize arabokinase as demonstrated by an increased $Q_{O_2}$ during incubation. Simultaneously Landman and Spiegelman (71) isolated a lactose positive mutant of *B. megaterium* and devised a stabilizing medium for protoplasts which permits synthesis of β-galactosidase. Virtually all of the enzyme forming capacity is recovered in the protoplasts. When supplemented with amino acids, hexose-diphosphate and inducer, they synthesize enzyme at rates comparable to intact cells. The β-galactosidase formed has been isolated in soluble form and purified. These preparations are amenable to enzymatic resolution, their enzyme forming activity being suppressed by RNAse. This treatment does not destroy them physically but selectively removes 80 % of the RNA.

It is evident that the search for a system which would permit the further experimental probing of protein synthesizing systems is at present in an exciting but preliminary stage. There seems little doubt, however, that a new era is being opened which will ultimately permit a description in chemically defined terms of the nature of the protein synthesizing machinery.

## SUMMARY AND CONCLUDING REMARKS

We have here surveyed the data which has accumulated on the phenomenon of 'enzymatic adaptation', with particular emphasis on the efforts of the past decade. In view of the complexity of the problem posed initially and the difficulties which could easily have hindered understanding, or led to irrelevant confusion, the progress which can be recorded is satisfying.

Operationally diverse disciplines have provided the data from which a picture of the enzyme forming mechanism has evolved. The kinetic, biochemical, and

genetic information on induced enzyme production all lead to and can be interpreted in terms of one model. They suggest that the enzyme forming system is a complex between RNA, inducer, and enzyme.

The problem has been brought to the point where further questions must be posed in terms of the chemical structures and reactive interrelation of the components identified. From the experiments reviewed in the last section, it would appear that the systems needed for the experimental resolution of precisely such questions are now on the way to development.

It seems likely at the present writing that the next decade will provide the necessary answers. Attention can then be profitably turned to the problem which initiated much of the work described, *i.e.*, what is the nature of gene function ?

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## Contribution à la discussion du rapport de S. Spiegelman intitulé « The present status of the induced synthesis of enzymes »

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(*Reçu le* 25 *novembre* 1955)

Les travaux de Brachet et de ses collaborateurs sur le rôle du noyau dans la synthèse des protéines conduisent à des conclusions qui s'accordent bien avec celles que M. Spiegelman vient de présenter, en ce qui concerne les relations de l'acide désoxyribonucléique (ADN) avec le système formateur d'enzymes.

Nous avons en effet montré avec Brachet (1) que $^{14}CO_2$ s'incorpore aussi vite dans les protéines des fragments anucléés d'*Acetabularia mediterranea* que dans celles des fragments contenant un noyau. Ce n'est que 2 ou 3 semaines après la section de l'algue que l'incorporation diminue dans les fragments anucléés. On pouvait en conclure que le système assurant l'incorporation de l'anhydride carbonique dans les protéines est largement indépendant du noyau. Vanderhaeghe (2) a pu préciser par des dosages directs de protéines sur des lots d'algues coupées, qu'une synthèse nette de protéines se produit à une vitesse normale ou même accrue dans les fragments anucléés pendant 15 à 20 jours ; après quoi la synthèse nette cesse dans les fragments anucléés, alors qu'elle se poursuit linéairement chez les nucléés.

Enfin, nous avons observé avec Brachet (3) qu'il est possible d'induire par l'eau oxygénée la formation de catalase chez *Acetabularia mediterranea*. Nous n'avons malheureusement pas réussi à maîtriser tous les facteurs dont dépend la synthèse induite de l'enzyme : alors que plusieurs cultures d'algues nous avaient donné invariablement une formation induite de catalase, il fut impossible l'année suivante d'obtenir des adaptations nettes. Quoi qu'il en soit, chaque fois (22 expériences) que la catalase s'est formée chez les fragments nucléés contenant un noyau, elle est apparue en quantités comparables chez les fragments anucléés.

Les résultats discutés par M. Spiegelman dans son rapport montraient que la synthèse d'ADN n'est pas une condition nécessaire de la synthèse d'enzymes induits. Les expériences de Brachet et de ses collaborateurs montrent que la présence du noyau (donc celle de l'ADN) n'est pas nécessaire à la synthèse des protéines cytoplasmiques. Nous conclurons donc que le matériel génétique nucléaire ne participe pas « personnellement » à la synthèse des protéines du cytoplasme, qu'il n'est pas un constituant du système formateur d'enzymes.

Des expériences récentes de Vanderhaeghe et Szafarz (4) montrent de plus que la synthèse nette d'acide ribonucléique (ARN) se poursuit à une vitesse normale ou même accrue dans les fragments anucléés d'*Acetabularia mediterranea* pendant quinze jours. Malkin (5) avait d'ailleurs observé que l'adénine-8-$^{14}C$ s'incorpore parfaitement dans l'ARN de fragments anucléés d'œufs d'oursins, et Kruh et Borsook (6) ont rapporté que le $^{14}C$ du glycocolle s'incorpore dans l'ARN d'un autre type de cellule anucléée, le réticulocyte.

Il faut en conclure que la synthèse d'ARN, comme celle des protéines cytoplasmiques, n'est pas dirigée directement par le matériel génétique nucléaire. Cependant, chez *Acetabularia meditarranea*, la synthèse nette de protéines et d'ARN n'est pas complètement indépendante du noyau, elle s'arrête en effet dans les fragments qui sont privés de noyau depuis 15 à 20 jours (2, 4, 7). Chez l'Amibe, l'énucléation exerce un effet beaucoup plus précoce sur l'ARN et sur certaines protéines cytoplasmiques, qui disparaissent des fragments anucléés bientôt après la séparation des fragments (8). Ceci indique qu'un agent produit par le noyau ou sous son contrôle immédiat, et qui s'épuise en son absence, est nécessaire pour la synthèse des acides ribonucléiques et des protéines cytoplasmiques. Nous ne connaissons pas la nature de cette substance ; il s'agit peut-être d'un métabolite banal et certains résultats de Brachet (8) suggèrent que ce pourrait être un coenzyme. Il est possible aussi que ce soit une substance transmettant un message plus complexe du matériel génétique au système formateur des acides ribonucléiques ou de protéines.

Soulignons enfin que les expériences d'énucléation ne nous renseignent nullement sur la synthèse des acides ribonucléiques et des protéines nucléaires ; il est évidemment possible que l'ADN joue un rôle direct dans la synthèse des protéines du noyau, ainsi que le suggèrent les expériences d'Allfrey (9) et peut-être aussi certains résultats de Gale (10) montrant que l'ADN influence la synthèse des protéines dans les staphylocoques brisés

En ce qui concerne le rôle de l'ARN, il est probable que l'inhibition de la synthèse des protéines par la ribonucléase dans les protoplastes (11, 12) et les homogénats (13, 14, 15) et sa restauration par l'ARN fournissent l'argument le plus convaincant de l'importance de ce dernier pour la synthèse des protéines. Il est extrêmement intéressant de constater de plus que la ribonucléase peut pénétrer dans certaines cellules vivantes, y bloquer la division cellulaire, la synthèse des protéines, et y bouleverser le métabolisme de l'ARN (16 à 29). Dans le cas des racines d'Oignon et des Amibes, il est même possible de bloquer la croissance par la ribonucléase, puis de la rétablir dans une certaine mesure en ajoutant de l'ARN (8, 17, 18, 19). La ribonucléase est donc un outil de choix pour l'étude de la fonction de l'ARN *in vivo*, et peut-être un agent capable d'enrayer la croissance normale ou pathologique.

Les expériences de M. Spiegelman indiquent que la formation d'enzymes induits ne se poursuit que si la synthèse d'ARN est possible. Gale et Folkes (30), et Creaser (31) ont noté de leur côté une incorporation d'uracile dans l'ARN pendant l'induction d'enzymes. Nous avons observé des faits semblables (32) pour l'incorporation d'adénine, d'hypoxanthine et d'uracile marquées dans l'ARN de la levure « au repos » (mutant petites colonies) pendant la formation induite de catalase, cytochrome *c* et cytochrome peroxydase. Si cette incorporation accrue reflète la synthèse nette d'ARN spécifiques ou la modification sélective de certaines molécules d'ARN, celles-ci doivent être plus fortement marquées que les autres et il doit être possible de les isoler. Nous avons fractionné des nucléoprotéines solubles de levure par précipitation au sulfate d'ammonium à pH 6. La vitesse d'incorporation de l'adénine-8-$^{14}$C est accrue bien plus dans certaines fractions que dans d'autres pendant l'induction d'enzymes. Ces premières observations sont donc favorables à l'idée d'une synthèse d'acides nucléiques spécifiques ou d'un remaniement sélectif de certains ARN. L'effet de l'induction sur l'incorporation d'adénine n'est pas affecté par la *para*-fluoro-phénylalanine aux concentrations auxquelles cet acide aminé inhibe presque complètement la synthèse induite de catalase. Ceci montre que la synthèse (ou la modification) de l'ARN n'est pas une conséquence de l'apparition de protéines nouvelles ; elle pourrait être une condition de la formation des enzymes induits. Soulignons toutefois que la levure contenait déjà une quantité de catalase et de cytochrome peroxydase facilement dosable avant l'induction : nos expériences n'excluent donc pas la possibilité que l'enzyme lui-même soit un constituant du « système formateur de l'enzyme » ou qu'il soit nécessaire à l'édification, à l'activation ou au maintien de ce système dans la cellule, conformément à l'hypothèse que M. Spiegelman vient de présenter dans son rapport.

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# The biosynthesis of porphyrins (*)

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(*Received* 26 *March* 1955)

Although the porphyrin molecule may be considered to be a rather complex molecule, it will be demonstrated that the cell accomplishes the synthesis of the porphyrin from two relatively simple compounds, glycine and succinate. A study of the biosynthesis of protoporphyrin, the porphyrin moiety of hemoglobin, has demonstrated that 'active' succinate, arising from the citric acid cycle, condenses on the α-carbon atom of glycine to form α-amino-β-ketoadipic acid which then decarboxylates to yield δ-aminolevulinic acid (figure 1). Condensation of two moles of the latter compound gives rise to a common precursor pyrrole, porphobilinogen, and four moles of the pyrrole then condense to form the porphyrin structure. On decarboxylation and dehydrogenation of the acetic and propionic acid side chains, protoporphyrin is formed. Although most of the studies were con-

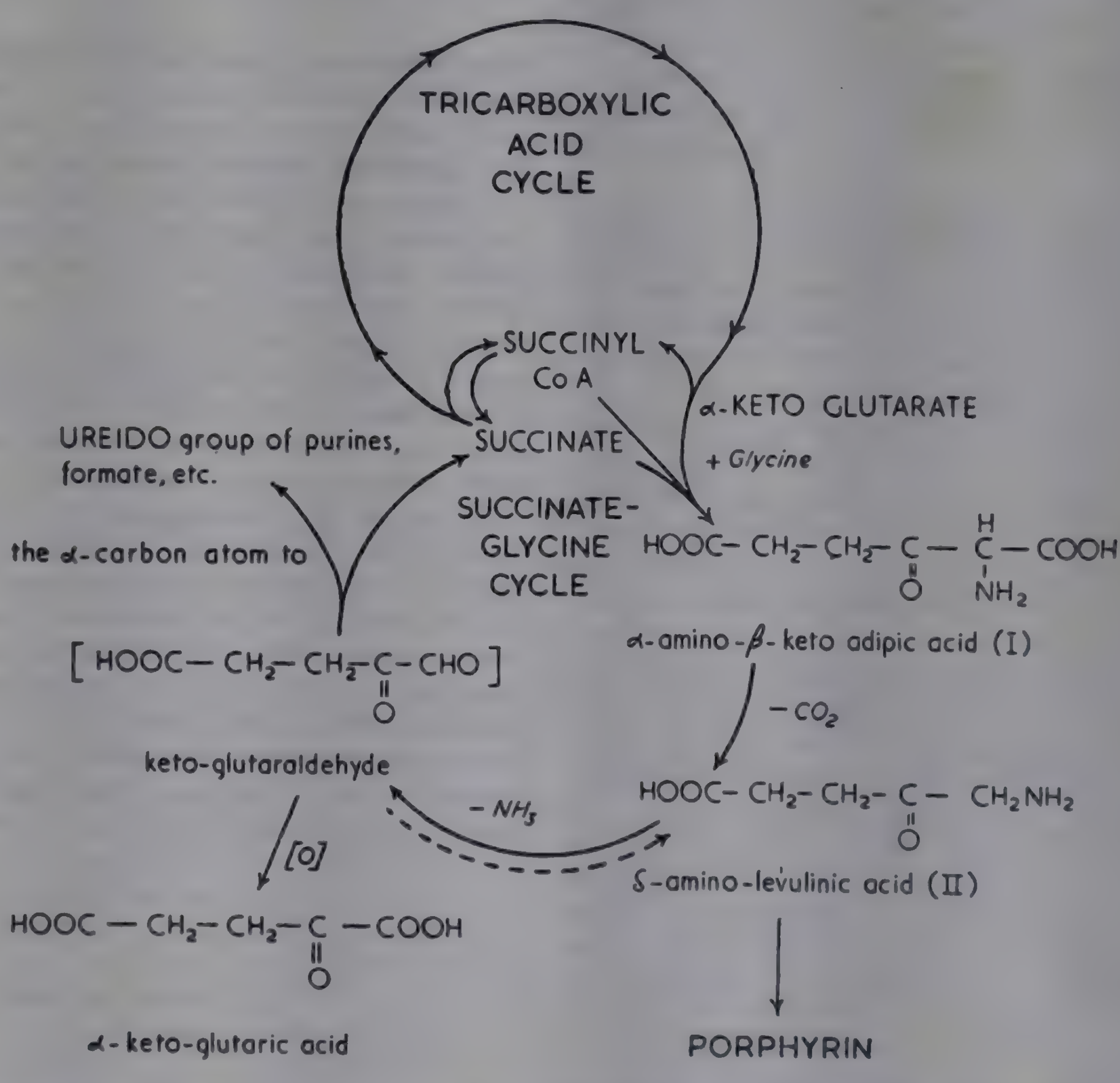


FIG. 1. — Succinate-glycine cycle : a pathway for the metabolism of glycine.

(*) This work was supported by grants from the National Institutes of Health, United States Public Health Service (RG-1128, C6), from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, from the Rockefeller Foundation and from the Williams-Waterman Fund.

cerned with the synthesis of protoporphyrin, it appears, from other studies, that the steps which were elucidated for the synthesis of protoporphyrin are universal in nature and that all porphyrins including chlorophyll in all different types of cells (bacteria, plant, avian, mammal) are synthesized by the same pathway. The different porphyrins arise by modification of the side chains in the β-positions on the pyrrole unit.

It may be well to point out that the biological system used in most of our experiments, dealing with the biosynthesis of protoporphyrin, was the nucleated red blood cells of the duck and preparations (homogenates and cell-free extracts) from these erythrocytes. It was found in 1948 (1) that duck erythrocytes incubated, *in vitro*, with isotopically labeled precursors of protoporphyrin synthesized labeled heme. It may be well now to consider the experiments which have led to the elaboration of the steps in porphyrin synthesis which are given in outline above.

In 1945 it was found that the nitrogen atom of glycine is the nitrogenous precursor of protoporphyrin (2). Since the nitrogen of glycine was equally utilized for the methyl and vinyl bearing pyrroles and for the methyl and propionic acid pyrroles (3, 4) we concluded, that in the synthesis of protoporphyrin, a common precursor pyrrole is first formed and the latter compound is the source of all four pyrrole rings of the porphyrin. This conclusion was well substantiated by subsequent experimental findings.

It appeared reasonable to expect that since the nitrogen atom of glycine is specifically utilized for porphyrin synthesis that the carbon atoms of this amino acid might also be involved. It was soon found that whereas the α-carbon atom of glycine is indeed utilized for porphyrin synthesis (5, 6, 7, 8), the carboxyl group was not (6, 9). This latter negative finding was an important clue in the elucidation of the mechanism by which glycine and succinate condense. However, on incubation of duck erythrocytes with doubly labeled glycine ($^{15}NH_2$-$^{14}CH_2$-COOH) it was found (6, 7, 8) that the dilution for the nitrogen atom was twice that for the α-carbon atom, that is, for every nitrogen atom utilized two carbon atoms from the α-carbon atom of glycine entered the porphyrin molecule. Therefore, it would appear that eight carbon atoms of the porphyrin molecule arise from the α-carbon atom of glycine since the four nitrogen atoms of the porphyrin are derived from glycine. In order to definitely establish that eight carbon atoms of the porphyrin are indeed derived from the α-carbon atom of glycine and if so to locate the positions of these carbon atoms in the porphyrin molecule, to gain some insight into the mechanism of porphyrin synthesis, we developed a chemical degradation procedure of protoporphyrin whereby each carbon atom from a particular position in the porphyrin could unequivocally be isolated (7, 10). On degrading protoporphyrin synthesized from glycine-2-$^{14}$C it was found indeed that eight carbon atoms are derived from the α carbon atom of glycine and the positions were located ; the four methene bridges (7, 8) and one in each pyrrole (7) (figure 2). It will be noticed that the carbon atoms in the pyrrole rings, derived from the α-carbon atom of glycine are in the α-position under the vinyl and propionic acid side chains. This finding

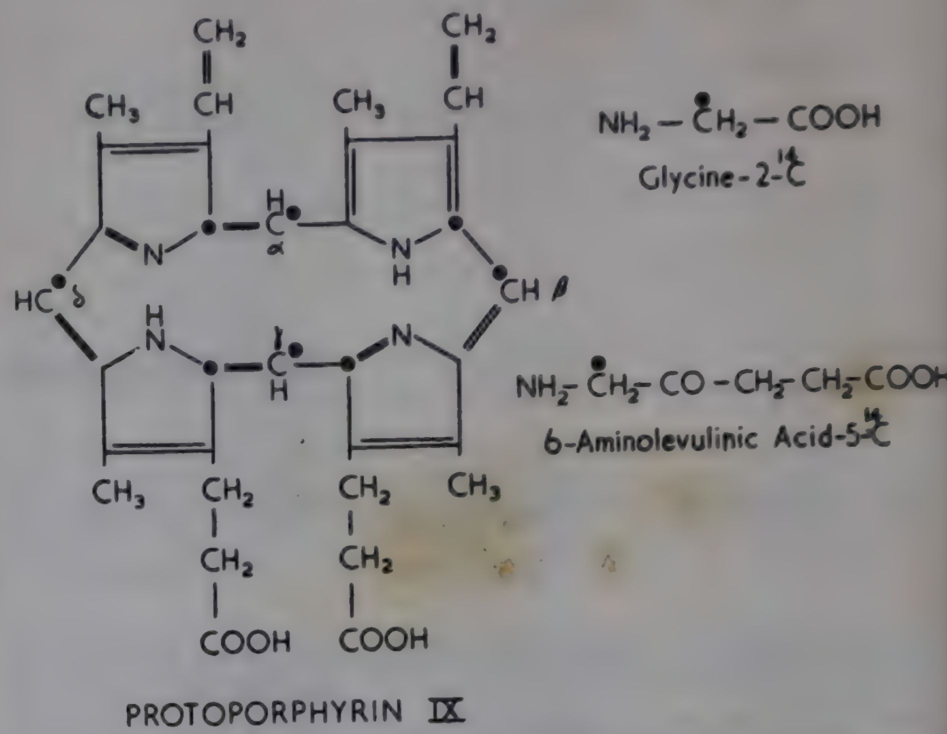


FIG. 2. — The carbon atoms of protoporphyrin which arise from the α-carbon atom of glycine and from the δ-carbon atom of δ-aminolevulinic acid.

supported the suggestion of a common precursor pyrrole first being formed and led to the suggestion that the vinyl side chains arose from propionic acid side chains by decarboxylation and dehydrogenation.

Having accounted for eight carbon atoms of protoporphyrin, the origin of the remaining twenty-six carbon atoms remained to be determined. It was found by Bloch and Rittenberg (11) that on administration of deuterioacetic acid ($CD_3COOH$) to a rat, the hemin isolated contained deuterium. This indicated that some of the side chain carbon atoms, at least, were derived from the methyl group of acetate since these are the only carbon atoms bonded to hydrogen.

In order to determined the extent of utilization of acetate for porphyrin synthesis and to locate all the carbon atoms which may be derived from acetate, duck blood was incubated separately with $^{14}$C methyl labeled acetate and with $^{14}$C carboxyl labeled acetate and the resulting $^{14}$C labeled hemin samples degraded by the method mentioned above. It was found that all the remaining twenty-six carbon atoms were derived from acetate (10). Further from the $^{14}$C labeling pattern among these carbon atoms it was concluded that the

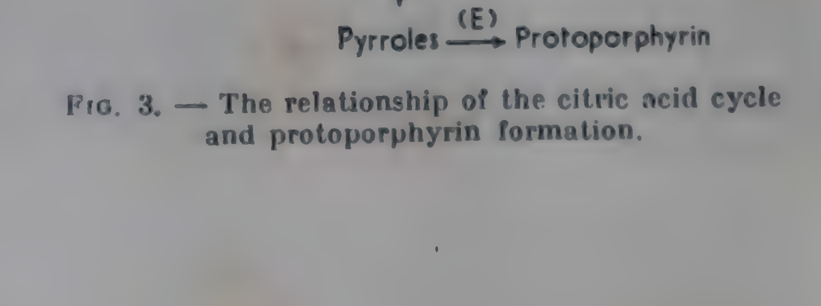


FIG. 3. — The relationship of the citric acid cycle and protoporphyrin formation.

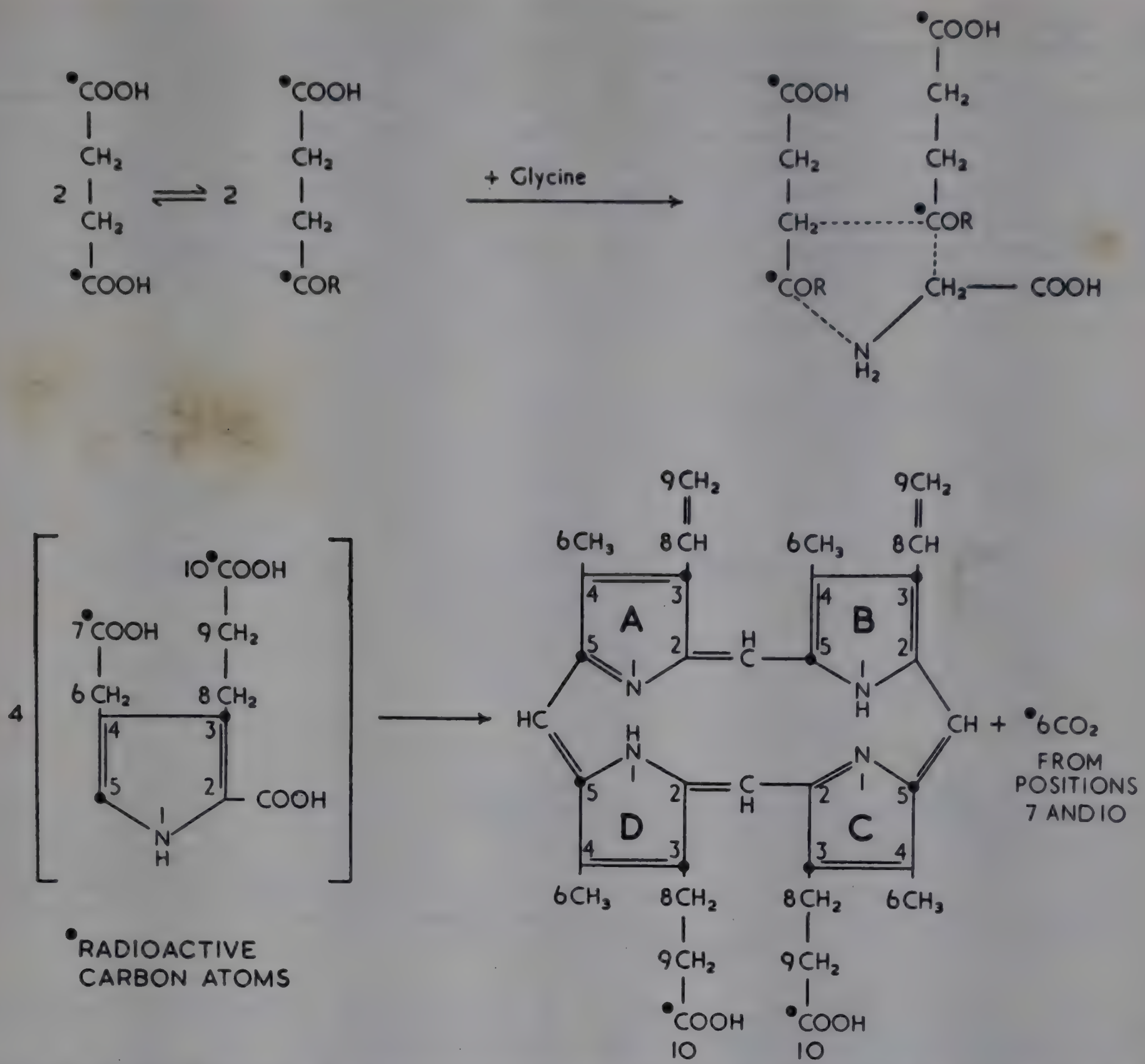


FIG. 4. — The position of succinate in protoporphyrin and the labelling pattern obtained in protoporphyrin synthesized from succinate-1,4-$^{14}C$.

acetate was utilized by being converted to a four carbon atom unsymmetrical compound *via* the citric acid cycle and that two moles of this compound condensed, in some manner, with glycine to form the precursor pyrrole. The pyrrole first formed bore acetic and propionic side chains in its β-positions (10) (figures 3 and 4). Direct documentation of these conclusions was obtained by studying the utilization of $^{14}C$ succinate (12), $^{14}C$ α-keto-glutarate and $^{14}C$ citrate (13) for porphyrin formation. In each case the predicted carbon atoms of the porphyrin contained the $^{14}C$. In figure 4, the over-all positions of glycine and succinate in the pyrrole unit and in the protoporphyrin are outlined and also in this figure are given the ten $^{14}C$ carbon atoms in protoporphyrin which are derived from the carboxyl group of succinate. These positions were theoretically predicted and experimentally determined (12).

It then became of interest to find the mechanism by which the 'active' succinate and glycine combine to form the pyrrole unit of the porphyrin. It was realized that in the initial condensation of glycine and succinate the whole molecule of glycine is involved since in all experiments in which glycine-2-$^{14}C$ was the substrate the carbon atom in the pyrrole ring and the methene bridge carbon atom (figure 2) had the same $^{14}C$ activity and no derivative of the α-carbon atom of glycine ($CH_3OH$, $H_2CO$, $HCOOH$, $CH_3NH_2$) could substitute for glycine. These findings led us to the conclusion that the same derivative of glycine was utilized for the pyrrole ring carbon atom and for the bridge carbon atom even though the bridge carbon atom was no longer attached to the nitrogen atom of glycine as is the ring atom. On consideration of the possible method of condensation of succinate and glycine, which would give rise to a product from which a pyrrole could reasonably be made, the mode of condensation must also take into consideration a mechanism by which the carboxyl group of glycine is detached from its α-carbon atom, subsequent to the initial condensation, for the carboxyl group of glycine is not utilized for porphyrin synthesis. The condensation of succinate on the α-carbon atom of glycine to form α-amino-β-ketoadipic acid (figure 1) would appear to be in agreement with all the experimental findings and conclusions. The compound formed, being a β-keto

acid, could then readily decarboxylate and thus provide a mechanism by which the carboxyl group of glycine is detached from its α-carbon atom subsequent to the initial condensation of the whole molecule of glycine with succinate. Further, the product of decarboxylation would be δ-aminolevulinic acid and condensation of two moles of the latter compound, by a Knorr type of condensation (figure 5) would give a reasonable mechanism for formation of a pyrrole in which the α-carbon atom of glycine would be distributed in the positions pre-

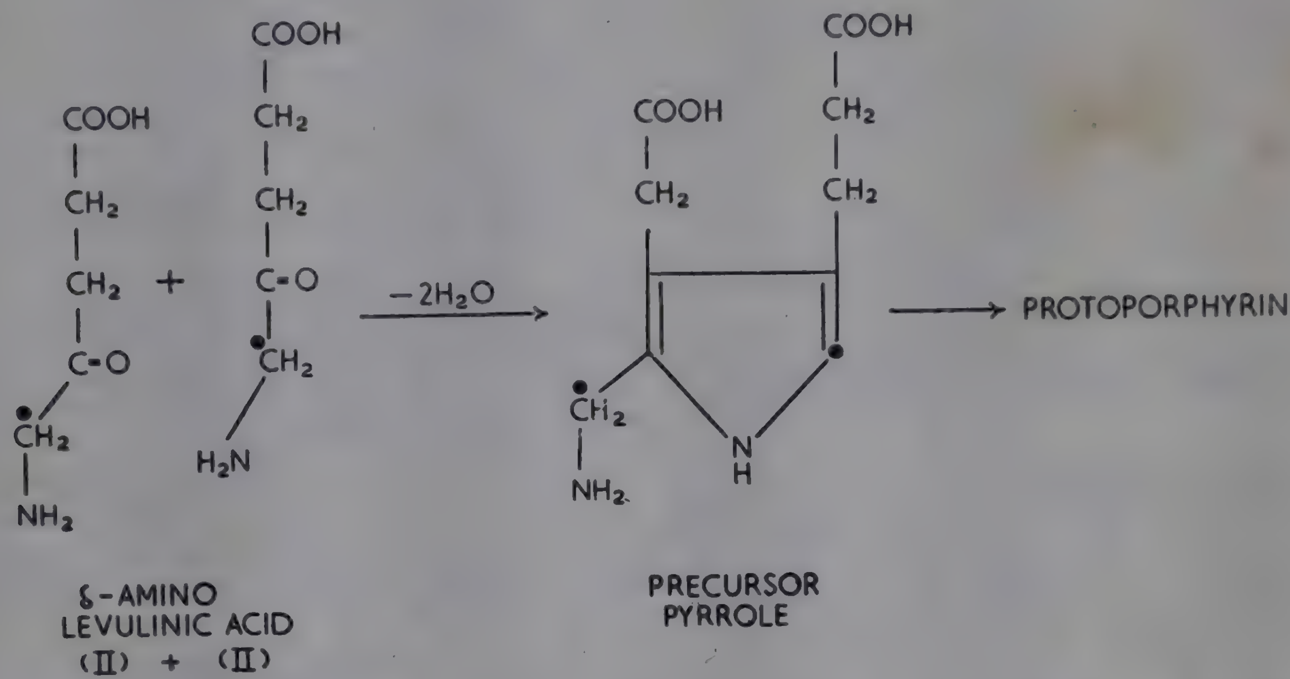


FIG. 5. — The mechanism for the formation of the monopyrrole, porphobilinogen, by condensation of two moles of δ-aminolevulinic acid. The carbon atoms bearing the closed circles (.) were originally the α-carbon atom of glycine.

viously observed. To test this hypothesis, δ-aminolevulinic acid was synthesized and its utilization for porphyrin synthesis studied (14, 15).

In the initial experiments, unlabeled δ-aminolevulinic acid was added to duck red blood cell hemolysates along with either $^{14}$C-labeled glycine of $^{14}$C-labeled succinate. The radioactivities of the hemin samples isolated in these experiments were compared with those obtained from controls in which the unlabeled δ-aminolevulinic acid was omitted. The rationale for these dilution-type of experiments is as follows : if δ-aminolevulinic acid is an intermediate formed from the condensation of glycine and succinate, any labeled δ-aminolevulinic acid formed from these labeled substrates will be diluted by the added unlabeled compound, and consequently this should be reflected in the lowered radioactivity of the hemin samples synthesized in the presence of unlabeled δ-aminolevulinic acid. It can be seen from table I that the hemin samples made in the presence of unlabeled δ-aminolevulinic acid contained less $^{14}$C than those of the controls made either from $^{14}$C-labeled glycine or succinate (14, 15). These results which are in full agreement with the hypothesis, can also be explained, however, by the possibility that δ-aminolevulinic acid is acting not as a diluent but as an inhibitor of heme synthesis. To rule out the latter possibility, the δ-aminolevulinic acid added in experiment 2 (table I) was labeled with $^{15}$N. It can be seen that whereas the incorporation of $^{14}$C from the glycine was lowered, there was a comparatively large incorporation of $^{15}$N into the porphyrin, thus demonstrating that the lowered $^{14}$C activity of the hemin sample was due to dilution rather than inhibition. Further proof that δ-aminolevulinic acid is a result of the condensation of glycine and succinate was obtained by incubating red blood cell hemolysates with glycine-2-$^{14}$C and unlabeled δ-aminolevulinic acid, and subsequently isolating the δ-carbon atom. In such an experiment it was found that the formaldehyde liberated upon periodate oxidation of a crude fraction containing δ-aminolevulinic acid was highly radioactive.

It now remained to establish more directly the utilization of δ-aminolevulinic acid for porphyrin formation. The compound was synthesized with $^{14}$C in the δ-carbon atom, and its utilization was compared with that of glycine. It can be seen from table II that the hemin synthesized from an equimolar amount of δ-aminolevulinic acid was about 65 times more radioactive than hemin synthesized from glycine. More rigorous proof that δ-aminolevulinic acid is indeed the precursor for porphyrin synthesis was obtained by degrading a hemin sample synthesized from δ-aminolevulinic acid-5-$^{14}$C and from δ-aminolevulinic acid-1,4-$^{14}$C.

The δ-carbon atom of the former compound should label the same carbon atoms of protoporphyrin as those which we have previously found to arise from the α-carbon atom of glycine, since according to the hypothesis the latter carbon atom is the biological source of the δ-carbon atom of δ-aminolevulinic acid. Furthermore the δ-aminolevulinic acid-1,4-$^{14}$C should label the same carbon atoms of protoporphyrin found to arise from the carboxyl groups of succinate since from figures 1 and 4 these carbon atoms arise from succinate.

It can be seen from table III that the same $^{14}$C distribution pattern was found in protoporphyrin synthesized from δ-aminolevulinic acid-5-$^{14}$C as from glycine-2-$^{14}$C ; 50 % of the $^{14}$C activity resides in the pyrrole rings and

TABLE I.

*Comparison of $^{14}C$-activities of hemin samples synthesized from glycine-2-$^{14}C$ (0.05 mC/m-mole) or succinic acid-2-$^{14}C$ (0.05 mC./m.-mole) in the presence and absence of non-radioactive δ-aminolevulinic acid*

| Experiments | Substrates | | | Isotope concentration in hemin | |
|---|---|---|---|---|---|
| | $^{14}C$-labeled | $^{15}N$-labeled | Unlabeled | $^{14}C$ (c.p.m.) | $^{15}N$ (atom % excess) |
| 1 | Glycine-2-$^{14}C$ (0.05 m-mole) | — | — | 125 | — |
| | Glycine 2-$^{14}C$ (0.05 m-mole) | — | δ-aminolevulinic acid (0.05 m-mole) | 15 | — |
| 2 | Glycine-2-$^{14}C$ (0.05 m-mole) | — | — | 230 | — |
| | Glycine-2-$^{14}C$ (0.05 m-mole) | δ-aminolevulinic acid (0.05 m-mole) | — | 48 | 0.21 |
| | — | Glycine (0.33 m-mole) | — | — | 0.06 |
| 3 | Succinate-2-$^{14}C$ (0.1 m-mole) | — | — | 660 | — |
| | Succinate-2-$^{14}C$ (0.1 m-mole) | — | δ-aminolevulinic acid (0.1 m-mole) | 180 | — |

The isotopic concentrations of the $^{15}N$-labeled substrates were 34 atom % excess $^{15}N$. In each of the experiments the volume of the hemolyzed preparation was 30 ml. Unlabeled succinate (0.1 m-mole) was added to the flasks in which labeled glycine was the substrate, and unlabeled glycine (0.33 m-mole) was added to the flasks in which labeled succinate was the substrate. Each flask contained 1 mg. of iron (ferric).

50 % in the methene bridge carbon atoms (see figure 2) (15, 16).

Also it can be seen from table IV that the same $^{14}C$ distribution pattern was found in protoporphyrin synthesized from δ-aminolevulinic acid-1,4-$^{14}C$ as from succinate-1,4-$^{14}C$; ten carbon atoms are equally radioactive, 40 % of the $^{14}C$-activity resides in pyrrole rings *A* and *B*, 60 % of the activity resides in pyrrole rings *C* and *D* and the carboxyl groups contain 20 % of the $^{14}C$ activity (see figure 4) (17).

Thus all the carbon atoms of protoporphyrin are derived from δ-aminolevulinic acid. The role of δ-aminolevulinic acid in porphyrin synthesis was also actively pursued by Neuberger and Scott (18), and just subsequent to our initial finding they published a confirmatory paper and further confirmation was published by

TABLE II.

*Comparison of $^{14}C$-activities of hemin samples synthesized from glycine-2-$^{14}C$ (0.05 mC/m-mole) and δ-aminolevulinic acid (0.05 mC/m-mole)*

| $^{14}C$-Substrate | Other additions | $^{14}C$-Activity of hemin (c.p.m.) |
|---|---|---|
| Glycine-2-C (0.05 m-mole) | Succinate (0.1 m-mole) | 333 |
| δ-aminolevulinic acid (0.05 m-mole) | — | 23 000 |
| δ-aminolevulinic acid (0.05 m-mole) | Glycine (0.33 m-mole) Succinate (0.1 m-mole) | 21 000 |

TABLE III.

*Distribution of $^{14}C$-activity in protoporphyrin synthesized from δ-aminolevulinic acid-5-$^{14}C$ and from glycine-2-$^{14}C$*

| Fragments of porphyrin | Molar activity (%) in fragments of porphyrin synthesized from | |
|---|---|---|
| | δ-amino-levulinic acid-5-$^{14}C$ | Glycine-2-$^{14}C$ |
| Protoporphyrin . . . . . . . . . . | 100 | 100 |
| Pyrrole rings *A* + *B* (methylethylmaleimide) . . . . . . . . . . | 24.5 | 24.6 |
| Pyrrole rings *C* + *D* (hematinic acid) . . . . . . . . . . | 25.2 | 25.3 |
| Pyrrole rings *A* + *B* + *C* + *D* . . | 49.7 | 49.9 |
| Methene brigde carbon atoms. . . | 50.3 | 50.1 |

TABLE IV.

*Distribution of $^{14}C$-activity in protoporphyrin synthesized from δ-aminolevulinic acid-1,4-$^{14}C$ and from succinate-1,4-$^{14}C$*

| Fragments of porphyrin | Molar activity (%) in fragments of porphyrin synthesized from | |
|---|---|---|
| | δ-amino-levulinic acid-5-$^{14}C$ | Succinic acid-1,4-$^{14}C$ |
| Protoporphyrin | 100 | 100 |
| Pyrrole rings $A + B$ (methylethylmaleimide) | 38.0 | 39.4 |
| Pyrrole rings $C + D$ (hematinic acid) | 61.5 | 59.5 |
| Pyrrole rings $A + B + C + D$ | 99.5 | 98.5 |
| Carboxyl groups | 20.4 | 20.5 |

Dresel and Falk (19). Furthermore, it may be well to point out that the theoretical formulation of the structure of the precursor pyrrole (14) is the same structure which was determined for porphobilinogen by Westall (20) and by Cookson and Rimington (21), a compound excreted in the urine of patients with acute porphyria. The utilization of δ-aminolevulinic acid for porphyrin formation adds to the certainty that porphobilinogen (22) is an intermediate in porphyrin synthesis. Although these findings make α-amino-β-ketoadipic acid an obligatory intermediate and we have found experimentally that this β-keto is indeed an intermediate. Injection of δ-aminolevulinic acid or the diethyl ester of α-amino-β-ketoadipic acid gives rise to the urinary excretion of porphobilinogen (23).

The condensation of 'active' succinate and glycine to form δ-aminolevulinic acid subsequently, thus far appears to require the partially intact structure of the red blood cell. It has been found that whereas δ-aminolevulinic acid can be converted to protoporphyrin in either an homogenized preparation or in a cell-free extract, the conversion of succinate and glycine to porphyrin takes place only with intact cells or with those cells which have been hemolyzed with water (16). Homogenized preparations obtained in a blendor are no longer capable of synthesizing protoporphyrin from succinate and glycine.. It would appear that on homogenization the functional activity of only those enzymes of the system that are involved in the condensation of succinate and glycine is lost. However, the finding that δ-aminolevulinic acid can be converted to protoporphyrin in a cell-free extract opened up the possibility that soluble enzymes, concerned with each of the steps in this conversion, could be isolated.

Indeed, it was subsequently and independently found in three different laboratories that a highly purified protein fraction from ox liver (24), duck erythrocytes (25) and from chicken erythrocytes (26) can convert δ-aminolevulinic acid to porphobilinogen. In our laboratory we obtained a highly purified fraction from duck blood which on incubation with δ-aminolevulinic acid-5-$^{14}C$ produced labeled porphobilinogen. Since the porphobilinogen is presumably synthesized from two moles of δ-aminolevulinic acid (figure 5), its molar radioactivity should be twice that of the δ-aminolevulinic acid used as the substrate. The molar radioactivities of the substrate, δ-aminolevulinic acid, and of the product, porphobilinogen, were found to be $242 \times 10^3$ c.p.m. and $487 \times 10^3$ c.p.m. respectively. This finding demonstrates experimentally the utilization of two moles of δ-aminolevulinic acid for porphobilinogen formation. Further evidence that porphobilinogen is an intermediate in protoporphyrin synthesis was obtained by incubating equal volumes of the cell-free extract of duck erythrocytes with equimolar amounts of δ-aminolevulinic acid (0.018 mC/millimole) and with the enzymatically synthesized radioactive porphobilinogen (0.036 mC/millimole) and subsequently isolating the hemin and determining its radioactivity. The radioactivities of the hemin samples synthesized from δ-aminolevulinic acid and from the porphobilinogen were 92 c.p.m. and 85 c.p.m. respectively, after a two-hour incubation, and 350 and 336 c.p.m. respectively after a fifteen-hour incubation period (25). This letter result is in agreement with the findings of Falk, Dresel and Rimington (22) and of Bogorad and Granick (27).

Although no evidence has yet been obtained concerning the biological mechanism of conversion of the monopyrrole to the tetrapyrrole structure, several suggestions have been advanced (27, 28). We would like to suggest still another possibility which may explain the distribution of the α-carbon atom of glycine or the δ-carbon atom of δ-aminolevulinic acid in the porphyrin molecule of the I and III series. This mechanism is based on the synthetic mechanism of dipyrrole and tetrapyrrole formation demonstrated by Corwin and Andrews (29), and by Andrews, Corwin and Sharp (30).

Condensation of three moles of the precursor pyrrole (porphobilinogen) or of a closely related derivative, would lead to a tripyrrylmethane compound, as schematically represented in figure 6. The tripyrrylmethane then breaks down into a dipyrrylmethane and a monopyrrole. The structure of the dipyrrylmethane is dependent on the place of splitting. An **A** split would give rise to dipyrrylmethane **A**, and a **B** split would give rise to dipyrrylmethane **B**. Condensation of two moles of dipyrrylmethane *A* would rise to a porphyrin of the I series, while condensation of a mole of *A* and a mole of *B* would give rise to a porphyrin of the III series. In the formation of the porphyrin of the III series it can be seen from figure 6 that it is necessary to lose a one-carbon atom compound since there are three aminomethyl side chains and only two are required to condense the two dipyrroles to the porphyrin structure. If the mechanism similar to that outlined in figure 6 is concerned with porphyrin synthesis it would appear that this one-carbon atom compound given off could well be formaldehyde. Consistent with this idea is our finding (15) that on the conversion of porphobilinogen to porphyrins either by heating under acid conditions (20) or by enzymatic conversion in cell-free extracts (16) formaldehyde was formed. This was established by heating or incubating porphobilinogen, labeled with $^{14}C$ in the aminomethyl group, and subsequently isolating radioactive formaldehyde as the dimedon derivative.

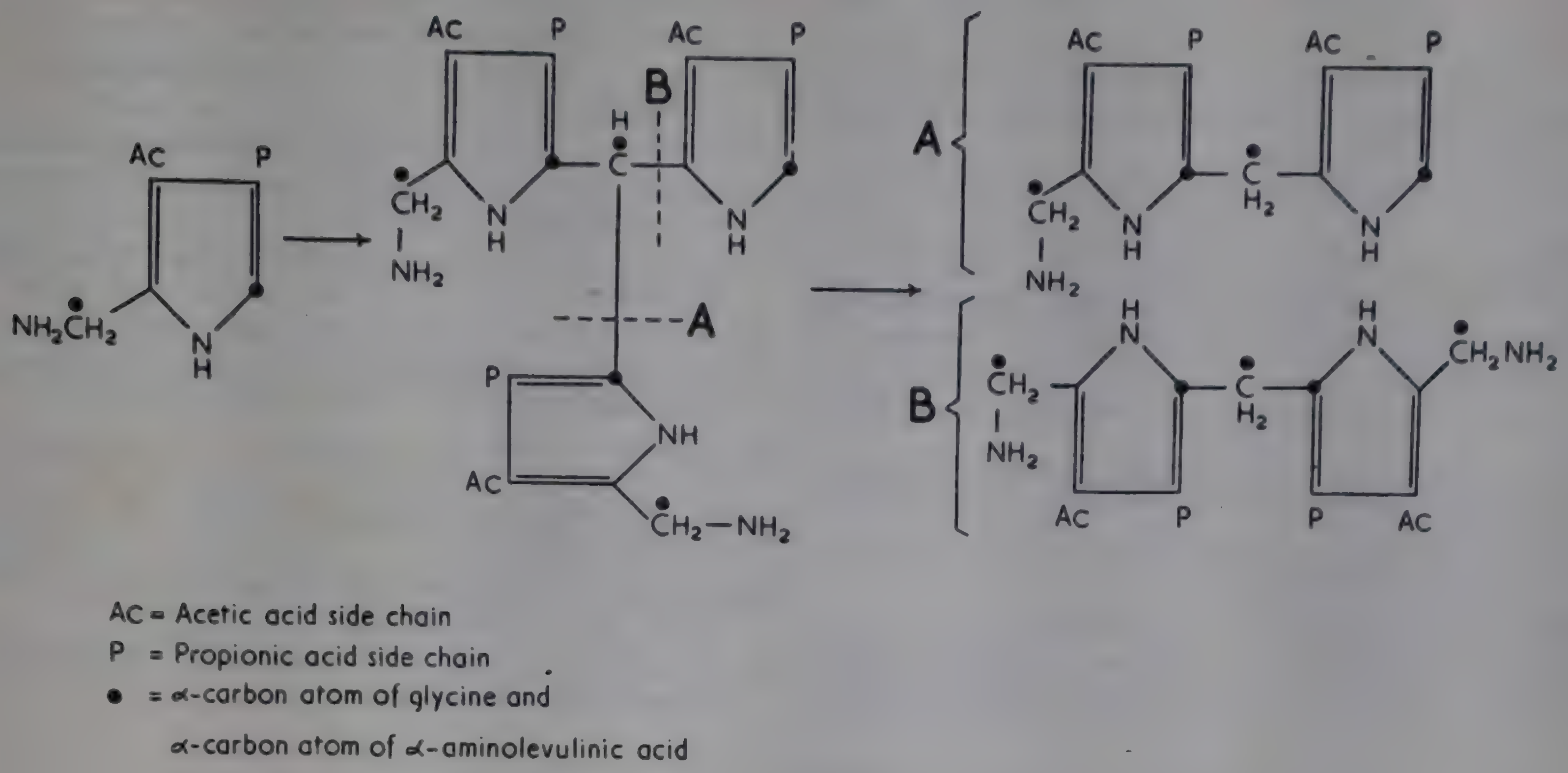


FIG. 6. — A mechanism of porphyrin formation from the monopyrrole.

It would appear that on conversion of porphobilinogen to porphyrins, formaldehyde from the aminomethyl group, is formed and that any postulated mechanism should take this into consideration. It is difficult at present to establish the structure of the intermediate etrapyrrole compounds which are formed prior to the formation of protoporphyrin. However, we would like to suggest that these intermediate tetrapyrrole compounds may be the more highly reduced state, containing methylene bridge carbon atoms rather than methene bridge carbon atoms, and consequently uroporphyrin and coproporphyrin are oxidized products of the intermediates.

The biosynthetic pathway for porphyrin synthesis, given above, may, from a more general viewpoint, be looked upon as merely one aspect of glycine metabolism. The α-carbon atom of glycine besides being utilized for porphyrin synthesis is also known to participate in the synthesis of several other compounds : the ureido groups of purines, the β-carbon atom of serine, methyl groups and for formic acid. It would appear that these different compounds and porphyrins may be related *via* a metabolic pathway of glycine. If indeed these mentioned compounds and porphyrin synthesis are related through a series of reactions occurring with glycine, then an intermediate utilized for porphyrin synthesis may have the same metabolic pattern as is known for glycine. If the succinate-glycine cycle proposed in figure 1 (14) were the pathway by which all the compounds are related then specifically the δ-carbon atom of δ-aminolevulinic acid should have the same metabolic spectrum as the α-carbon atom of glycine. In a study carried out in ducks and rats it was found that indeed the δ-carbon atom of this aminoketone is utilized for the ureido groups of purines, for the β-carbon atom of serine, for the methyl group of methionine and is also converted to formic acid. Thus, it has been demonstrated, that glycine is metabolized *via* this pathway (31).

The succinate-glycine cycle also provides a mechanism by which the carbon atoms of glycine can be oxidized to carbon dioxide. The formation of α-amino-β-ketoadipic acid and subsequent decarboxylation of this β-keto acid would yield one mole of carbon dioxide. This mole of carbon dioxide was originally the carboxyl group of glycine. If the postulated ketoglutaraldehyde or some other derivative can be converted to α-ketoglutaric acid, a pathway is provided for the conversion of the α-carbon atom of glycine to carbon dioxide, for the α-carboxyl group of the ketoglutarate was originally the α-carbon atom of glycine. α-ketoglutaric acid isolated from a cell-free extract of duck red blood cells which were incubated with δ-aminolevulinic acid-5-$^{14}$C contained radioactivity.

The condensation of glycine with 'active' succinate provides a pathway whereby glycine can be oxidized to carbon dioxide and the intermediates produced in the cycle drawn off for the synthesis of other compounds. This is similar to the citric acid cycle, in which another two-carbon compound is oxidized to carbon dioxide and intermediates are produced which can be drawn off for synthesis. In the succinate-glycine cycle, succinate is the catalyst instead of oxaloacetate.

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# The metabolism of delta-aminolaevulic acid 'in vivo' and properties of the delta-aminolaevulic dehydrase


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(*Received* 20 *May* 1955)

Dr. Shemin has given us a brilliant exposition of the present state of our knowledge of the biogenesis of porphyrins in the development of which he has played the leading part. The evidence for the position of α-amino β-oxoadipic acid and that of δ-aminolaevulic acid in this scheme rests almost entirely on *in vitro* experiments (1, 2, 3). I shall not describe our own experiments with such systems which are less complete than those done by the Columbia group, but fully confirm those presented by Dr. Shemin; I shall report in more detail experiments done with the whole animal.

## In vivo *metabolism of ALA*

These experiments (4, 5) show that δ-aminolaevulic acid (ALA) behaves as a precursor of porphyrins in man and in the rat. Thus, administration of ALA gives rise to the excretion of porphobilinogen (PBG) in the urine, and to excretion of fairly large amounts of protoporphyrin in the faeces. If labelled ALA is given, the faecal stercobilin has a high isotope content, but the protoporphyrin isolated from circulating haemoglobin is poorly labelled. These findings and the large amount of ALA which is excreted unchanged in the urine, permit the following general conclusions (6) :

(*a*) ALA has a low renal threshold, *i.e.* it is either poorly or not at all reabsorbed by the tubules.

(*b*) ALA administered enterally or parenterally is converted by tissues other then the bone marrow or circulating red cells to porphyrins or haem compounds.

(*c*) Administered ALA is not efficiently used for porphyrin formation by the erythropoietic system partly owing to its fast excretion by the kidney, partly owing to its degradation by extramedullary tissue and probably to some extent due to its slow rate of penetration into the erythroblast.

The experiments on which these conclusions are based were carried out mainly by Dr. J. J. Scott in partial collaboration with Dr. N. Berlin and Professor C. H. Gray. When $^{15}N$-labelled ALA was given to rats, about 35 to 65 % of the $^{15}N$ was excreted in the urine, the proportion excreted varying with the dose. Most of this excretion occurs in the first six hours.

The findings with ALA differ from those obtained in similar experiments with $^{15}N$-labelled α-amino acids such as glycine in two respects. Most of the $^{15}N$ is not in the urea, but in the residual nitrogen, *i.e.* the non-urea non-ammonia fraction. This nitrogen consists mainly or entirely of ALA-N, as shown by parallel experiments in which unlabelled ALA was used. Thus, giving similar doses of ALA and using colorimetric methods of estimation devised by Dr. Shuster in my laboratory (7), it was found that 30 to 60 % was excreted unchanged in the urine. The second unusual feature is the high value for the $^{15}N$ atom % excess in the ammonia fraction. It is possible that ALA is deaminated

(*) Read at the Congress by J. J. Scott.

in the kidney, but further work is required to establish this point and to exclude the possibility that this finding represents an artifact.

One of the substances present in the urine after ALA administration is porphobilinogen (PBG). This has been demonstrated both by colorimetric and chromatographic methods and by isolation of crystalline PBG. The efficiency of this conversion is a function of the dose, as shown by experiments both in the rat and in man. Log (m-moles PBG excreted) increases linearly with log (m-moles ALA injected) up to a saturation dose of ALA, beyond which the PBG excreted remains constant.

We next examined the behaviour of [1,4 — $^{14}C_2$] ALA in man. As expected, about one third of the radioactivity appeared in the urine in the first few hours (figure 1); most of this was accounted for by the

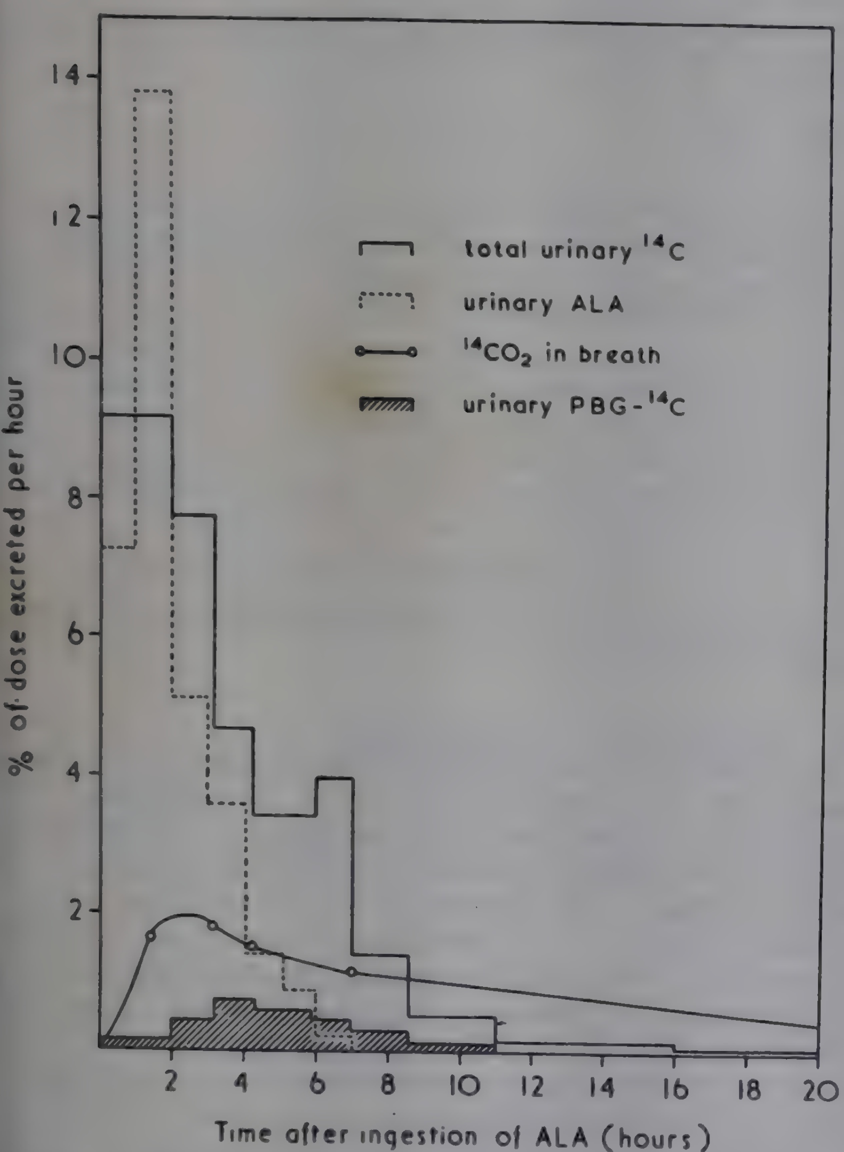


Fig. 1. — Rates of excretion of radioactivity in urine and breath after giving [1,4 — $^{14}C_2$] ALA (Reproduced by kind permission of the Ciba Foundation, London).

excretion of unchanged ALA, but significant contributions were also made by PBG and $NaHCO_3$. About 15 % of the dose was excreted as $CO_2$ in the breath and 16 % in the faeces. Thus, after four days 80 % of the administered radioactivity was eliminated in the breath, urine and faeces.

The faeces contained a very large quantity of porphyrin which consisted almost entirely of protoporphyrin. This porphyrin is found normally in human faeces, in quantities normally less than 0.5 mg./day, but the very large amount of 13 mg. found in a 24 hr. sample must be ascribed to the ingestion of ALA.

The dilution of the isotope in the various products (table I) shows very high relative activities for faecal protoporphyrin and urinary PBG. However, both are well below 100 % and thus indicate either that the ingested ALA has been diluted by endogenous ALA and/or that such a dilution has occurred from mixing with endogenous PBG and protoporphyrin.

TABLE I

*Isotope dilutions observed after ingestion of $^{14}C$ ALA*

| Compound | Starting activity | Activity when isolated as % of theoretical value if no dilution |
|---|---|---|
| δ - aminolaevulic acid (ALA) . . . . . . . . | **100** | ? |
| Urinary porphobilinogen | --- | 78.1 |
| Faecal protoporphyrin (corrected for loss of 6 $CO_2$) . . . . . . . | --- | 58.3 |
| Faecal stercobilin . . . | --- | 2.92 |
| Haem . . . . . . . . . | --- | 0.014 |

The activity of the stercobilin is low relative to that of PBG, but very high in comparison with the haem. This, together with earlier findings on the activity-time curve of stercobilin after administration of labelled glycine suggests that ALA is to some extent converted to a porphyrin or haem of a protein, such as catalase, which is rapidly metabolised to bile pigment. We have also, with the help of Captain Van Dyke, U.S.A.F., carried out some experiments with bile duct cannulated rats using the technique of Dr. Hardin Jones. About 30 min. after administration of ALA by stomach tube the bile began to show red fluorescence and the biliary porphyrin output did not return to normal until some time between 12 and 24 h. (6). The experiment showed that the increase in porphyrin was entirely due to protoporphyrin, whilst the output of coproporphyrin remained low and nearly constant. No porphyrin or ALA or PBG could be detected in the bile. PBG, but no free porphyrin, was present in the urine.

In man and rat, 'administered' ALA is a poor precursor of haemoglobin porphyrin, compared with glycine. It is, however, utilized by the intact organism about 100 times more effectively than succinate. The actual fraction of the dose of ALA converted to haemoglobin haem we find to be much the same in cockerels, rats and man, being between 0.1 and 0.3 %.

I may conclude this account of our *in vivo* experiments by mentioning that all human subjects who ingested ALA showed a marked erythema of the exposed parts of the body due apparently to skin-photosensitivity. This erythema resembled a sunburn and the severity of the symptoms was related to the dose of ALA.

### *The enzymic conversion of ALA to PBG*

Evidence for the existence of an enzyme which converts ALA to PBG was first obtained in avian red cells by Dresel and Falk (3) and this enzyme was presumably present in the porphyrin-synthesizing system of Shemin *et al.* (8). A purification of this enzyme and some of its properties were first described by Gibson *et al.* (9), but almost simultaneously two other independent communications (10, 11) appeared in which similar work was reported. The work I am describing has been mainly done by my colleague Mr. K. D. Gibson.

This enzyme, which we have called ALA dehydrase, has a wide distribution in nature (12). The liver is by far the richest source of all mammalian tissues we have examined, whilst kidney and bone-marrow have lower, but still fairly high activities. It is of interest that the activity in liver and kidney has been consistently increased by about 100 % on treatment with sedormid. The enzyme is also widely distributed in microorganisms. In liver the enzyme appears to be present exclusively in the soluble part of the cytoplasm.

The enzyme has been purified about 1000 times overall by conventional methods, consisting of acetone precipitation, heat treatment, $(NH_4)_2SO_4$ precipitation and adsorption on calcium phosphate gel. The final solution is straw-coloured and on electrophoresis at pH 8.4 gives a sharp and symmetrical peak (figure 2).

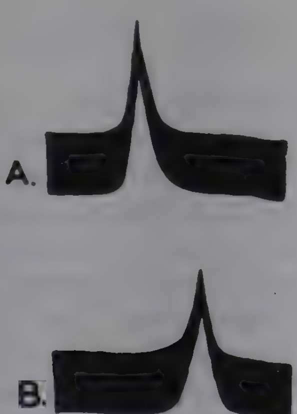


FIG. 2. — Electrophoresis of purified ALA dehydrase; scanning photograph; pH 8.42, ionic strength 0.2, 2 ½ hours.
A, ascending boundary; B, descending boundary.

The formation of PBG and the disappearance of ALA have been measured simultaneously and the results obtained (12) indicate that half a mole of PBG is formed for each mole of ALA disappearing.

The PBG produced in the enzymic reaction has also been isolated in cystalline form and identified chromatographically. The pH optimum of the enzymic reaction is 6.8 and the activity decreases sharply on the acid side and more slowly on the alkaline side (12).

The substrate specificity of the enzyme has not been extensively investigated. However, neither the higher homologue, ε-amino δ-oxohexanoic acid nor α,δ-diamino-γ-oxovaleric acid, which contains an additional amino group, give Ehrlich-positive compounds in the presence of the enzyme. It also appears that neither amino-acetone nor ethyl α-amino β-oxoadipate are acted upon by the enzyme. It would thus seem that the enzyme requires, apart from the aminoketone grouping, a carboxyl group at the right distance from the reacting part of the molecule. It would also seem likely that the carboxyl group attaches itself to or interacts with the enzyme during the reaction.

ALA dehydrase is almost certainly a sulphydryl enzyme. After the third step of the purification the addition of cysteine or glutathione is needed in order to obtain any activity. Ascorbic acid and dithionite are without effect. The relationship between concentration of GSH and activity is shown in figure 3.

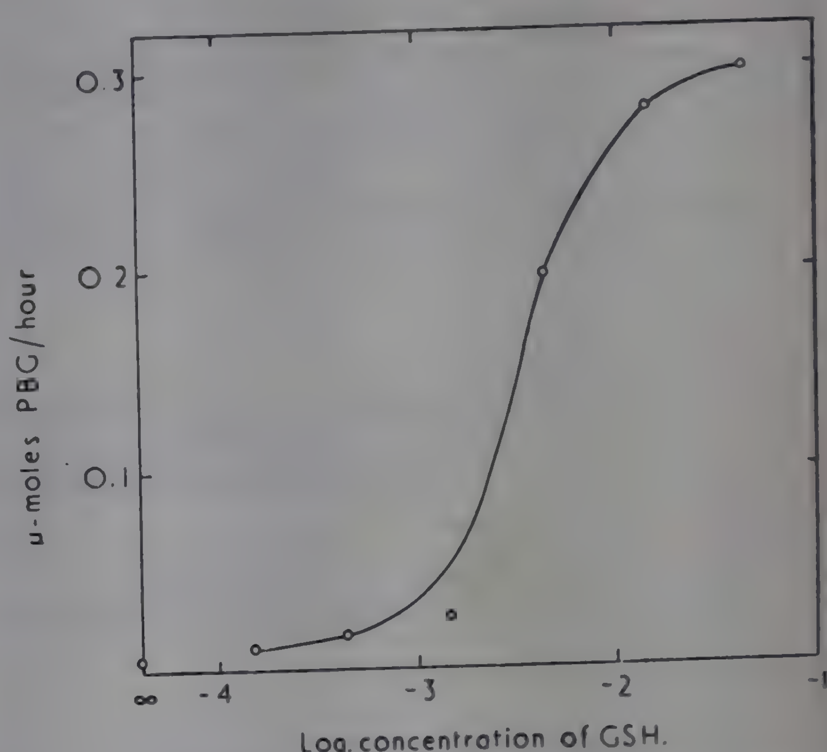


FIG. 3. — Activation of purified ALA dehydrase by GSH, at pH 6.8 and 38° C.

Iodoacetamide and *p*-chloromercuribenzoate inhibit completely at a molarity of $10^{-3}$; but addition of an equimolar amount of GSH abolishes this inhibition partly or almost completely. Sodium arsenite was without effect. No indication has so far been obtained of the presence of a prosthetic group. The ultraviolet spectrum of our purified preparation is that expected of an ordinary protein. Versene inhibits completely at $10^{-4}$ M, but no requirement for a metal has so far been discovered. The enzyme is inactive in the presence of tris-hydroxymethylaminomethane buffer ('tris') but is active in a mixture of 'tris' and phosphate buffer. However, there is no specific requirement for phosphate since the enzyme is also active in bicarbonate or arsenate.

The reaction which is catalysed by this enzyme is a condensation of two aliphatic compounds to give an aromatic compound. One C-C bond and one C-N bond are formed with the elimination of two molecules of water. It seems possible that the enzyme catalyses the formation of the C-C bond (10) and that the C-N condensation then follows spontaneously. Kinetic experiments have so far not been helpful in elucidating the reaction mechanism and it is also still uncertain whether the sulphydryl group is directly involved.

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## Studies on blood catalase

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(*Received* 2 *August* 1955)

The catalase activity in whole blood was determined in healthy adults and in patients with various diagnoses. The rapid titration technique was used. Hemoglobin, erythrocytes and reticulocytes were determined as well by means of the usual methods.

In healthy individuals the catalase activity was proportional to the hemoglobin content of the blood and to the number of red cells. There was, however, a considerable scattering both in this group and in the following one. For the healthy subjects the value $k \approx 1$/sec./ml. whole blood was found.

In cases of liver cirrhosis, sideropenic anemia, acute porphyria, porphyria cutanea tarda, some endocrine disorders, polycythemia, pernicious anemia, and hemolytic anemias the catalase activity was also proportional to the hemoglobin concentration and, but less so, to the number of red blood cells, with the relation given above ($k \approx 0.2$/sec./million of red cells/ml. of blood).

The catalase activity was independent of the number of reticulocytes per ml. of blood. It was followed during the treatment of a case of pernicious anemia with vitamin $B_{12}$. No significant change in catalase activity corresponding to a thirthy fold increase in reticulocyte number was seen.

## Chemische Grundlagen zur Biosynthese der Porphyrine

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(*Eingegangen am* 9 *September* 1955)

Die bewundernwerten Arbeiten von Dr. D. Shemin und seiner Mitarbeiter zur Suche eines Ausgangsstoffes der Biosynthese der Porphyrine und des Hämins führten zur Erkenntnis, dass δ-Aminolävulinsäure (ALA) aus Glykokoll und Bernsteinsäure aufgebaut wird und über einen Pyrrolbaustein in Porphyrin umgewandelt wird. Es ist sogar gelungen, durch Markierung mit Isotopen jedes einzelne C- und N-Atom des auf biochemischen Weg gebildeten Hämins einwandfrei den entsprechenden Atomen des Glykokolls und der Bernsteinsäure zuzuordnen. Damit ist die Voraussetzung geschaffen, den Reaktionsmechanismus der Biosynthese des Hämins und der Porphyrine und in Erweiterung auch wohl des Chlorophylls aufzuklären. Weitere wichtige Erkenntnisse und Bestätigungen verdanken wir vor allem den Arbeitskreisen um Neuberger und Rimington.

Die Isolierung und Aufklärung der Konstitution des Pyrrolderivates Porphobilinogen (PBG) durch Westall (1), Cookson und Rimington (2), sowie Kennard (3) bedeutet eine Bestätigung der Voraussagen. Von grosser Bedeutung ist der Nachweis der Biosynthese von PBG aus ALA, der Biosynthese von Hämin aus PBG, sowie der Bildung von Uroporphyrin I und III mit biochemischen, wie auch mit rein chemischen Methoden aus PBG.

Ausser der Fülle biochemischer Probleme ergeben sich nun aus den neuen Befunden auch zahlreiche rein chemische Fragen. So ist zu erklären : (*a*) Wie erfolgt der Uebergang von ALA in PBG ? (*b*) Welches ist der Weg der Biosynthese des Porphyrin-Systems ? (*c*) Wie werden sowohl Porphyrine der Reihe III wie auch der Reihe I als einheitliche Farbstoffe oder im Gemisch aus ALA gebildet ? Ein direkter Uebergang der Porphyrine beider Reihen ineinander ist aus einleuchtenden Gründen völlig ausgeschlossen. (*d*) Wie wird die Propionsäuragruppe zur Vinylgruppe des Hämins abgebaut ?

An Versuchen eine Hypothese des Reaktions-Meche-

nismus aufzustellen hat es nicht gefelht (4, 13, 16). Wir sind durch unsere Synthese des Uroporphyrin III, das wir als Hauptprodukt statt des zunächst erwarteten Uroporphyrin I aus der α-Oxymethylpyrrol-α-carbonsäure erhielten veranlasst worden, uns mit dem Problem auseinanderzusetzen (4).

Die ausführliche Mitteilung unserer Synthese haben wir zurückgestellt (5), da die Ergebnisse ausgedehnter Versuchsreihen über bestimmte Reaktionen einfacher Pyrrole und Pyrrolfarbstoffe abgewartet werden sollten, die für die Umsetzung der Pyrrole von allgemeiner Bedeutung sind. Wir sind heute in der Lage die Substitutionsreaktionen der Pyrrole, die für die Bildung der Pyrrolfarbstoffe massgebend sind, weitgehend und bis in die Einzelheiten zu verstehen (5).

## I

Die Bildung des Porphobilinogens aus 2 Mol ALA kann gedeutet werden nach dem Schema der Knorr'schen Pyrrolsynthese, wie dies auch von Shemin formuliert wurde. Dieser Pyrrolringschluss gelingt jedoch nur dann mit guter Ausbeute, wenn 1,3-Dicarbonylverbindungen mit Aminoderivaten von 1,3-Dicarbonylverbindungen kondensiert werden, erfordert also besonders aktive CO- und $CH_2$-Gruppen. Einfache Ketone und Aminoketone, und ein solches ist ALA, geben mit dieser Methode Pyrrole nur in geringer Ausbeute. Daher war zu vermuten, dass der Uebergang von ALA in PBG kein rein chemischer, sondern ein enzymatischer Vorgang ist. Neuberger hat dafür den überzeugenden Nachweis erbracht und ein Enzym, Aminolävulinsäure-dehydrase, nachgewiesen, das sehr spezifisch auf ALA eingestellt ist.

## II

Für den unmittelbaren Uebergang eines Pyrrols in Porphyrin haben wir ein Vorbild in der Umsetzung von Pyrrolen mit 2 unbesetzten α-Stellungen mit Ameisensäure (6, 7), oder mit Formaldehyd und dessen Derivaten (8). Noch näher kommt die Porphyrin-Synthese von Siedel und Winkler (9) über α-Oxymethylpyrrole. Dabei wurde bereits die überraschende Tatsache festgestellt, dass nicht etwa Gemische aller möglichen Porphyrine der 4 Reihen gebildet werden, sondern je nach dem β-Substituenten in der Hauptsache solche der Reihen I und daneben II. Wir erhielten dagegen, wie bereits bemerkt, Uroporphyrin III. Die Porphyrin-Bildung aus α-Oxymethylpyrrol-α-carbonsäure auf rein chemischen Weg erfordert bemerkenswerterweise ziemlich starke Mittel, hohe Temperatur oder Erwärmung mit starker Säure. Auch bei PBG wird, trotz der freien α-Stellung, Erhitzen mit verdünnter Säure benötigt.

Auch für die Biosynthese von Porphyrin aus PBG ist daher die Mitwirkung von Enzymen zu fordern; damit könnte eine Spezifität durch Lenkung in Richtung zur Porphyrin-Reihe III oder I verbunden sein. Zwei verschiedene Porphyrin-synthetisierende Faktoren sind daher von vorneherein wahrscheinlich. Dabei muss in beiden Fällen der Desaminierung eine wesentliche Rolle zukommen, aber auch die Aktivierung des Pyrrolkerns ist zu fordern.

Zunächst ist eine grundsätzliche Frage zu stellen. Kommt eine der Porphyrinsynthesen über Dipyrrylmethene oder eine Synthese über Dipyrrylmethane in Betracht? Alle Synthesen über Dipyrrylmethene, die formal Dehydrierungsprodukte der Dipyrrylmethane sind, erfordern bromierte Derivate und sehr energische Reaktionsbedingungen. Mehrere Bildungsweisen von Porphyrinen aus Dipyrrylmethanen verlaufen jedoch unter sehr milden Bedingungen. Daher kann mit fast völliger Sicherheit angenommen werden, dass Verbindungen vom Typ der Dipyrrylmethane Zwischenstufen der Biosynthese sein müssen.

## III

Eine Synthese einheitlicher Porphyrine der zentrosymmetrischen Reihe I ist verständlich durch unmittelbar gleichmässige Aneinanderreihung von 4 Molen PBG und Ringschluss oder wahrscheinlicher durch eine in 2 Schritten erfolgende Reaktion.

Shemin stützt sich mit seiner Hypothese der Bildung von Porphyrinreihe III und I auf ein Reaktionsschema Corwins (10, 11). Corwin versucht damit die Bildung symmetrischer Dipyrrylmethene statt der erwarteten asymmetrischen aus Pyrrolaldehyd und Pyrrol zu verstehen. Asymmetrische Dipyrrylmethene sind Zwischenprodukte der wichtigsten klassischen Porphyrinsynthesen H. Fischer's. Das Reaktionsschema betrifft die Anlagerung und Abspaltung von Pyrrol an Dipyrrylmethen. Diese Reaktionen haben wir jetzt klargestellt. Sie sind abhängig von den basischen Eigenschaften der betreffenden Pyrrole und den Aciditätsverhältnissen (12), im Gegensatz zur Ansicht Corwins; die Konstitution und die Einheitlichkeit der synthetischen Porphyrine sind nicht in Frage gestellt.

Der Vorschlag Shemins schliesst in Wirklichkeit einen viel komplizierteren Dehydrierungs- und Hydrierungsmechanismus ein und müsste korrekt wie folgt ausgeführt werden. Diese Einbeziehung der Tripyrrylmethane ist jedoch unnötig, und diese gekoppelten Oxydations- und Reduktionsprozesse sind äusserst unwahrscheinlich; die gleiche Ueberlegung gilt auch für die Hypothese von Bogorad und Granick (13), die ebenfalls ohne Berechtigung Dipyrrylmethane in Tripyrrylmethane übergehen lassen. Die Bildung der beiden Dipyrrylmethane ist auch verständlich, wenn man primäre Bildung von Formaldehyd annimmt.

Träger dieser Reaktionen und der meisten Pyrrol-Substitution-Reaktionen sind die Salze von Pyrrolen, die nach Treibs und Michl (14) als Pyrrolenin-Kationen zu formulieren sind (Abbildung 1). Nun dürfte durch die Aminogruppe die Salzbildung des Pyrrolkerns in PBG und damit auch die Reaktionsfähigkeit der α-Stellung gehemmt sein. Andererseits ist durch zalhreiche Reaktionen die Labilität von Br-, OH-, OR-, $NR_2$-Resten in der α-Methylgruppe erwiesen, was mit der Tendenz zur Salzbildung des Pyrrolkerns in Zusammenhang stehen muss. Alle diese Verbindungen reagieren unter der Wirkung von H-Ionen unter Abspaltung von Formaldehyd und Selbstkondensation, am leichtesten die OH-Verbindung, die die grösste Tendenz zur Pyrrolenin-Salzbildung haben sollte. Wahrscheinlich ist Desaminierung der erste Reaktionsschritt (*a*) zum Oxymethylderivat *III* (Abbildung 1) und nicht die gleichzeitig

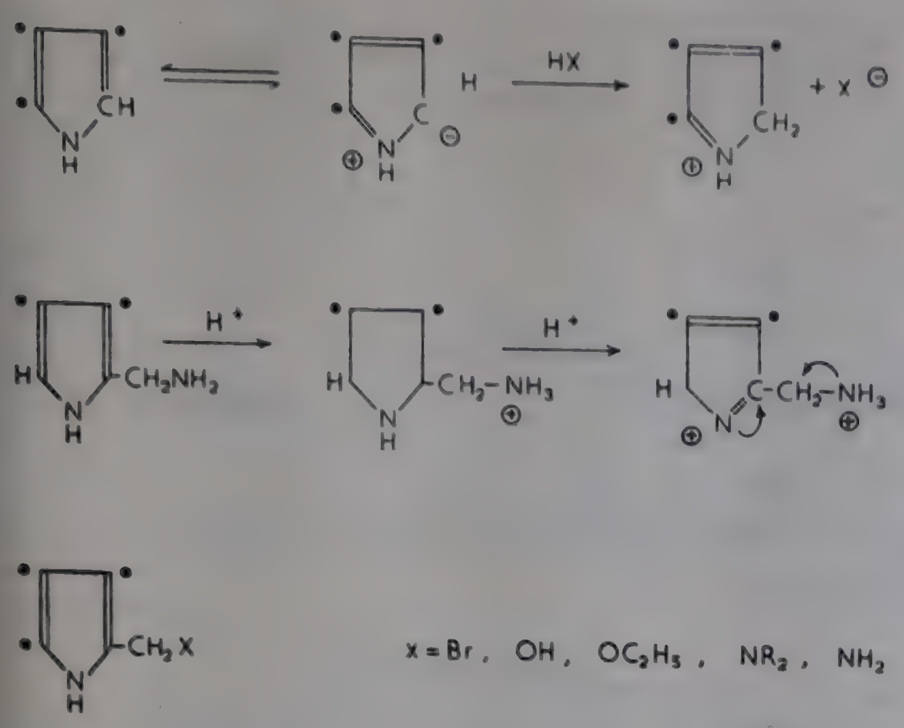


Abb. 1

Abspaltung von Formaldehyd und Ammoniak. Der Nachweis von Formaldehyd aus PBG mit Dimedon ist eine schöne Bestätigung, gestattet jedoch keinerlei Rückschlüsse auf den Reaktionsmechanismus. Dr. Falk hat nach einer Privatmitteilung die gleiche Nachweismethode angewandt. Das Oxymethylderivat *III* kann nun weiterhin Formaldehyd abspalten zum Pyrrol *IV* (*b*) eine reaktion, die in saurem pH-Gebiet reversibel ist, aber auch zur isomeren Oxymethylverbindung *V* führen kann. Andererseits wird Formaldehyd auch mit *III* nach (*c*) zum Dimethylolpyrrol *VI* reagieren. Dimethylolpyrrol *V* und α-freies Pyrrol *IV* müssen in äquivalenten Mengen auftreten. Auch die Bildung von Oxymethylaminomethylpyrrol ist in Erwägung zu ziehen, was aber keine grundsätzliche Änderung bedeutet, es könnten daraus Aminomethylpyrromethane entstehen. Oxymethylpyrrole kondensieren äusserst leicht mit solchen Pyrrolen, die freie Methingruppen besitzen, zu Dipyrrylmethanen und zwar bereits unter der Wirkung sehr schwacher Säuren.

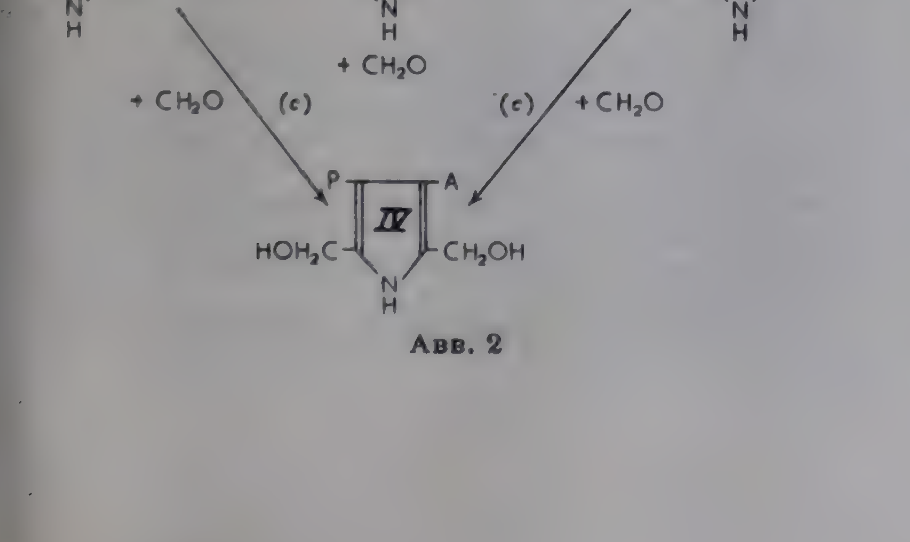


Abb. 2

Die Carbinole *I* und *IV* können demnach folgende Dipyrrylmethane *V*, *VI*, *VII*, *VIII*, *IX* und *X* bilden (abbildung 3). Bezieht man nun weiterhin auch das Carbinol *III* ein, so ergeben sich weitere Dipyrrylmethane, deren Formulierung unterbleiben soll. Während nun die Spaltung und Bildung der Oxymethylpyrrole ein reversibler Vorgang ist, sind die Dipyrrylmethane stabil und nicht mehr spaltbar. Alle diese Reaktionen der Oxymethylpyrrole sollten bei saurer Reaktion gleichzeitig ablaufen.

Die Dipyrrylmethane *V*, *VI*, *VIII*, *X* sind nun in Bezug auf die Anordnung der β-Substituenten gleich, sind einander demnach bei der Porphyrinsynthese gleichwertig. Kondensation von 2 Mol *V* führt nun zur Leukoverbindung von Uroporphyrin I. Die Kondensation von 1 Mol *VI* mit 1 Mol *VII* ergibt unter Formaldehydverlust die Leukoverbindung von Uroporphyrin III. Die Kombination von *V*, *VIII* und *X* mit *VI* gibt Porphyrin-Reihe III und Reihe I, Kombination der drei Dipyrrylmethane *V*, *VIII*, *X* mit *VII* ausschliesslich Reihe III. Diese Reaktionen sollten sogar bevorzugt sein, da die 3 Dipyrrylmethene freie α-Stellungen bereits besitzen. Auch die Kombination von *IX* mit *V*, *IX* mit *X* ergibt Reihe III.

Selbstkondensation von *IX* mit Formaldehyd *IX* mit *VII*, sowie die Einbeziehung der Dipyrrylmethane aus Pyrrol *III* gibt hingegen auch Anlass zur Bildung von Uroporphyrin II und IV.

Aus diesen Ableitungen kann gefolgert werden :

(*a*) Kommt es durch einen enzymatischen Prozess zur Reaktion (*a*) allein, ohne Bildung von Formaldehyd, so its das einzig mögliche Porphyrin Uroporphyrin I.

(*b*) Wird dann nach Reaktion (*b*) Formaldehyd abgespalten, so muss PBG auch andere Uroporphyrine bilden. Die Reaktionen (*a*) und (*b*), sowie die folgenden Kondensationsreaktionen können allein durch H-Ionen bewirkt werden und laufen in diesem Falle alle gleichzeitig ab. Das Ergebnis der Einwirkung von Säuren auf PBG muss daher ein Gemisch von Uroporphyrin sein, das viel Porphyrin der Reihe III, wenig I und wahrscheinlich sehr wenig II und IV enthält. Die Bildung von reinem Uroporphyrin I oder III aus PBG allein durch Säurewirkung ist daher unmöglich, ist aber bei enzymatischer Reaktion denkbar. Enzyme, die Reaktion (*b*) und die folgenden Kondensationsreaktionen katalysieren, dürften H-Ionen-Donatoren sein.

(*c*) Die grossen Verdünnungen in physiologischem Medium wirken entsprechend dem Ziegler'schen Verdünnungsprinzip der theoretisch möglichen Polykondensation entgegen, begünstigen demnach die Bildung der Leukoporphyrine.

(*d*) Die primär gebildeten Leukoporphyrine werden durch Oxydationsvorgänge zu den Porphyrinen oxydiert. Dipyrrylmethane und Leukoporphyrine sind durch die Ehrlich'sche Pyrrolreaktion nachweisbar (15), was bei der Unterscheidung von PBG zu beachten ist.

(*e*) Aus den bekannten chemischen Reaktionen folgt zwangsläufig, dass Porphyrinreihe III und I allein begünstigt sind. Die Suche nach Porphyrinen der Reihen II und IV als Nebenprodukte sollte nicht ohne Aussicht auf Erfolg sein.

(*f*) Für Hypothesen grundsätzlich anderer Wege zum Porphyrinsystem besteht bisher keine Notwendigkeit.

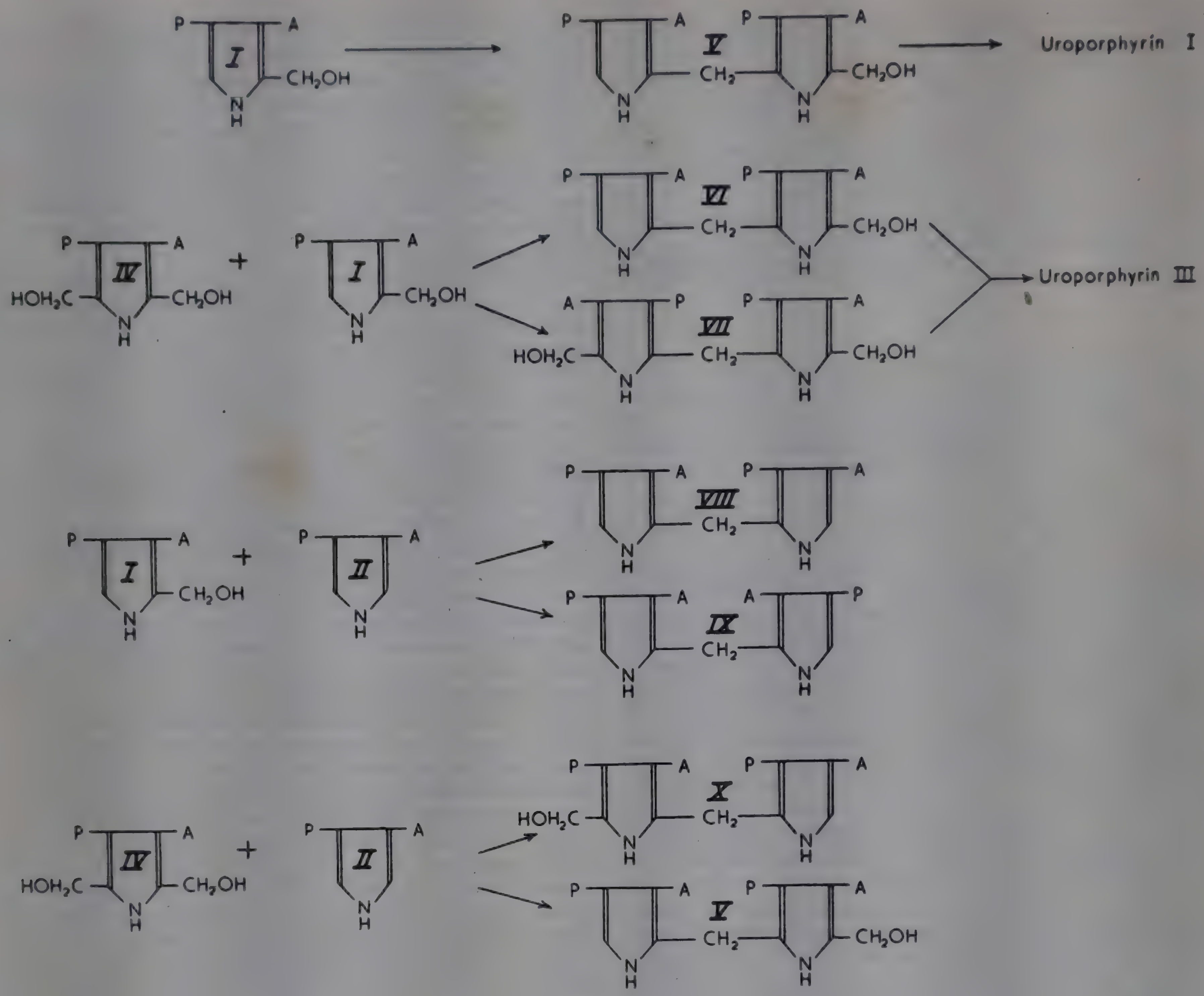


Abb. 3

IV

Der rein chemische Abbau von Propionsäuregruppen zu Vinylgruppen kann durch Analogie nicht belegt werden. Hier muss ein enzymatischer Prozess, Dehydrierung und Decarboxylierung, oder umgekehrt, ablaufen. Bisher sind noch keine Anhaltspunkte gegeben in welcher Stufe der Biosynthese diese Reaktionen vor sich gehen, beim 1-kernigen Pyrrol oder Dipyrrylmethan, deren Reaktionsfähigkeit dadurch wesentlich beeinflusst werden könnten, beim Leuko-Uroporphyrin III oder erst bei Uroporphyrin III. Die widerspruchsvollen Resultate verschiedener Bearbeiter der Frage of Uroporphyrin III eine Zwischenstufe der Hämin-Synthese ist und Unterschiede der *in vitro*- und *in vivo*-Versuche, können durch Störungen oder Ueberlastung des Enzymsystems bedingt sein. Hier müssen weitere Ergebnisse Aufklärung bringen.

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# The photosynthetic carbon cycle (*)

by M. CALVIN

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(*Received* 8 *April* 1955)

## INTRODUCTION

As you know, the problem of photosynthesis is the one of determining how the green plants store electromagnetic energy in a chemical form by performing this chemical transformation :

$$CO_2 + H_2O \xrightarrow{h\nu} (CH_2O)_x + O_2$$

converting the carbon dioxide and water into carbohydrates and molecular oxygen. This reaction has been separated into two pretty well distinct parts, both theoretically and actually physically : first, the quantum conversion process involving the splitting of water into some sort of reducing agent plus one-half mole of oxygen :

$$H_2O \xrightarrow{h\nu} [H] + [O]$$

followed by the reduction of carbon dioxide, using this reducing agent [H] to produce carbohydrates :

$$CO_2 + [H] \longrightarrow (CH_2O)_x$$

Now what I have to say this afternoon is concerned mostly with this last half of the operation, of which we have been able to draw a complete map, using carbon-14 tracers.

This is a work of a period of about eight or nine years and of a laboratory of fluctuating population and size.

## FIRST PRODUCTS

The essential character of the experiment is simple; to set up a plant in a steady state of photosynthesis, feed it carbon dioxide, with labeled carbon in it, and trace the path of carbon as it goes through a sequence of transformations. The way in which this has been done has been described a number of times. So I am going to go through quite rapidly the sequence of operations which lead to the results at the end. The steady state is set up by using green algae in a lollipop (figure 1)

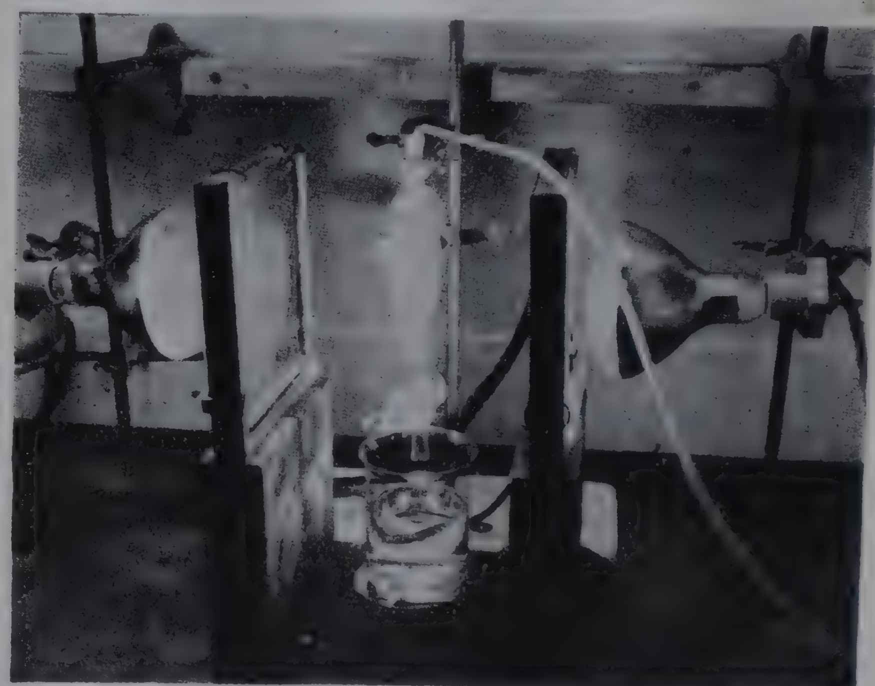


FIG. 1.

and the radiocarbon is fed in at a given time, over a period of some seconds to minutes. The algae are killed by dropping them into hot alcohol and then the alcohol extract is examined for radioactive compounds. This examination is performed by means of paper chromatography and radioautography; figure 2 shows the results of a 60-second experiment, and you see there are some twenty or more compounds formed from the radioactive carbon, and it is quite clear that 60 seconds is too long a time. We therefore shorten the time progressively; figure 3 shows a 10-second experiment and by this time one compound is predominant. In fact if the experiment is extrapolated back to zero time all the radioactive carbon turns out to be in the phosphoglyceric acid. Thus we have been able to identify the first compound into which carbon dioxide is incorporated by photosynthesis.

(*) Most of the work described herein has been documented in (1) « The Path of Carbon in Photosynthesis. XXI », by J. A. Bassham, A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson and M. Calvin, *J. Am. Chem. Soc.*, 1954, **76**, 1769, and (2) « Photosynthesis » by J. A. Bassham and M. Calvin, a chapter in *Currents in Biochemical Research* (edited by D. E. Green), to be published by Interscience Publishers, Inc. The new material given in this paper is the work of J. R. Quayle, R. C. Fuller, J. Mayaudon, K. Shibata, J. A. Bassham and D. F. Bradley and will be particularized at the proper point. The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

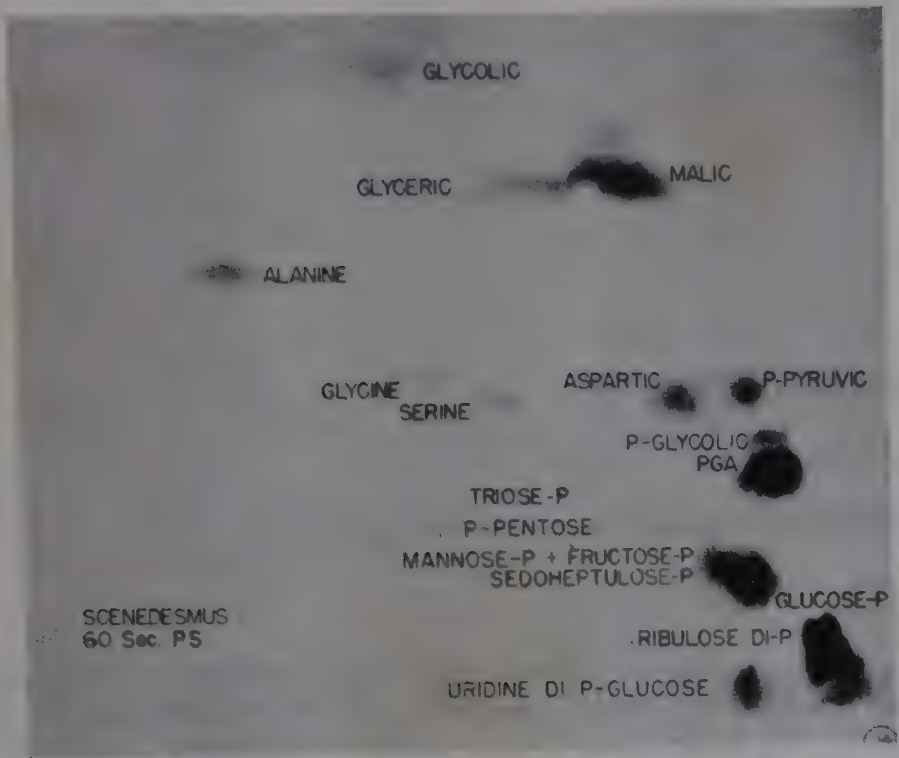



**Fig. 2.**

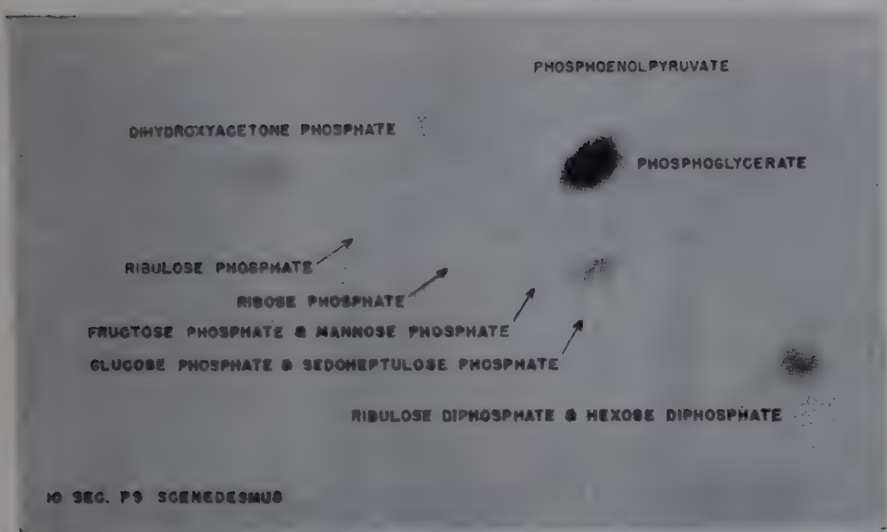



Fig. 3.

Figure 4 shows the distribution of the labeled carbon in the three carbon atoms of the glyceric acid obtained from the phosphoglyceric acid; this happens to be a 15-second experiment. Half of the activity is in the carboxyl group and the other half is equally split between the other two carbon atoms. From the same

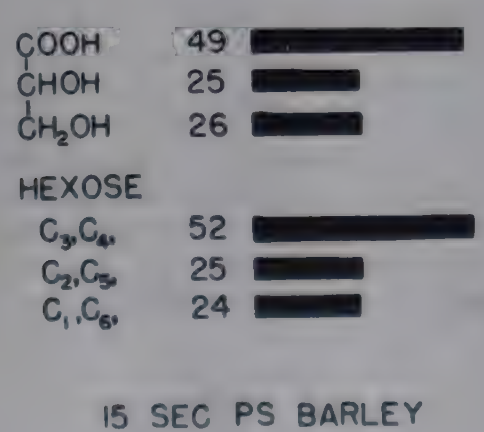



Fig. 4.

experiment a sugar molecule was obtained, and it also was degraded, and the distribution of carbon in it was found to be very much the same as it is in the three-carbon piece of glyceric acid. This immediately suggests that the six-carbon piece is made from the two three's by putting the two carboxyl groups together, which, of course, is simply a reversal of the well-known aldolase split of fructose diphosphate in the glycolytic sequence, a part of which is shown in figure 5. Here the glyceric

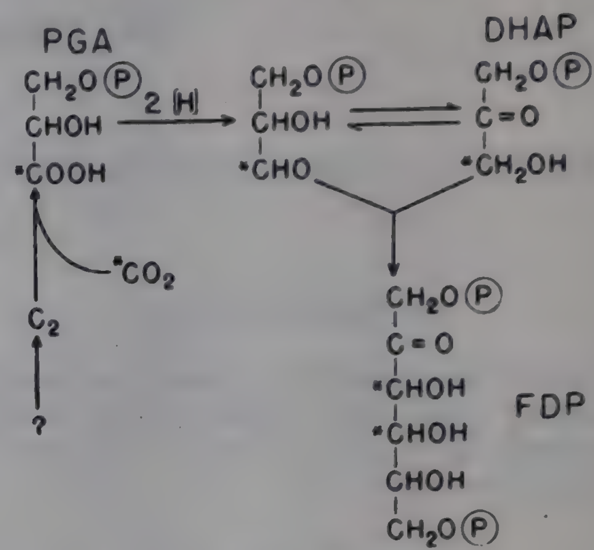



Fig. 5.

acid is reduced with the hydrogen from the photochemical reaction to glyceraldehyde, which is then isomerized to the ketotriose, and the two of them hooked together to give hexose. Thus, the two carbon atoms which were originally carboxyl finally fall in the middle of the hexose chain. It's quite clear that there must be something which accepts the carbon dioxide to form the glyceric acid and, furthermore, that that something must be regenerated from the PGA (phosphoglyceric acid), triose phosphates and hexose phosphates or something else formed from them.

*Sucrose synthesis*

In passing, it might be worth describing what we know about the fate of the fructose diphosphate and how it gets to the common table sugar sucrose. This was worked out by identifying several of the additional spots on the paper and showing their relationship to fructose phosphate and to sucrose. Figure 6 shows that relationship as we found it. Here is shown the phosphoglyceric acid, the fructose diphosphate and the various transformations which lead ultimately to glucose-1-phosphate. This reacts with uridine triphosphate to make uridine diphosphoglucose. This uridine diphosphoglucose (UDPG) is found on the paper, with glucose labeled very rapidly, and it then can react in either of two ways : either with fructose-1-phosphate to form sucrose phosphate, which was found on the paper, and then is phosphatased to sucrose, or it can react directly with free fructose to form sucrose in one operation. However, since we never see any free labeled fructose, the first of these alternatives appears to be the major pathway for the green leaves. And I might add that an enzyme performing the reaction

UDPG + fructose phosphate → sucrose phosphate

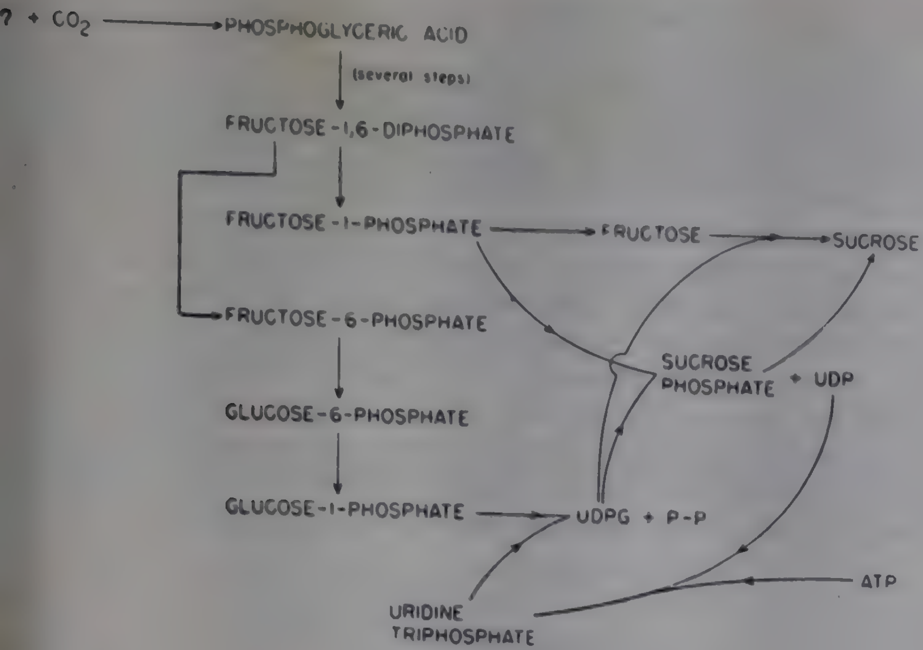



FIG. 6.

has recently been prepared in a partially purified state by Leloir in Argentina. Figure 7 shows the structural formula for the UDPG, and its reaction with fructose-1-phosphate, a sort of a double decomposition reaction giving uridine diphosphate and sucrose phosphate with the phosphate on the number 1 carbon atom of the fructose moiety. This, then, is finally taken off to give sucrose. This appears the common route to sucrose; it is one of the major synthetic reactions in agriculture and provides the substrate for a wide variety of other transformations.

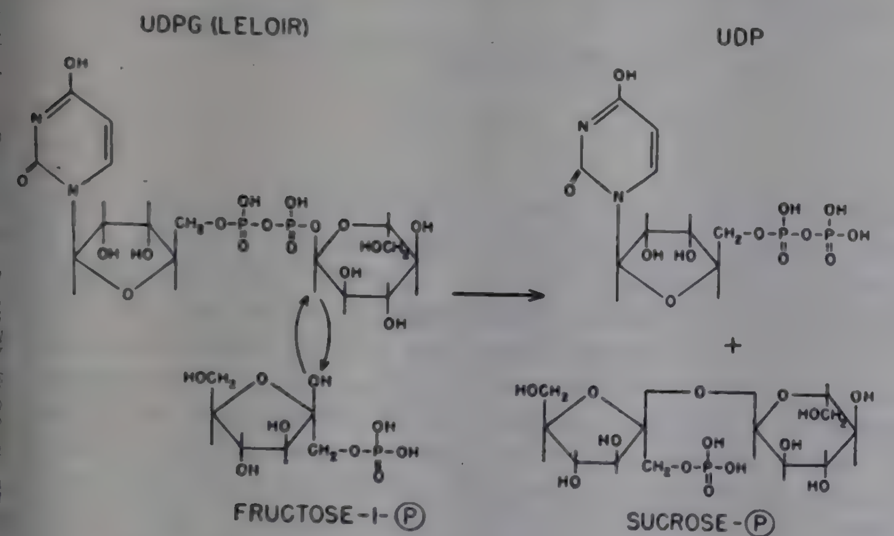



FIG. 7.

*The $C_5$ and $C_7$ sugars*

Let us return to the problem of where and how the acceptor of the carbon dioxide is created, or regenerated, rom these various other substances which we have een. In trying to pursue the original plan further, hat is, to see if we couldn't outline the sequence of teps which lead to the regeneration by simply taking ut the PGA and extrapolating back to zero time for he next compound which would appear at 100 % and which should be the next one in the sequence, we found t to be impossible. That is, three or four compounds xtrapolated out, suggesting that, not one was the next after PGA and triose, but several simultaneously formed from this one precursor (this triose precursor). Indeed, the compounds which showed up on further analysis, in addition to the six-carbon sugar, which you have seen, were a five-carbon and a seven-carbon sugar; the three-, five-, six- and seven-carbon sugars, then, were found. A detailed analysis of the distribution of radioactivity among the carbons of these sugars is shown in figure 8. Here beside the PGA, is the five-carbon sugar, ribulose diphosphate (RuDP); the seven-carbon sugar, sedoheptulose phosphate (SMP); and the skeleton of a six-carbon sugar, corresponding either to glucose or fructose (these are the major six-carbon sugars which we find). The stars give some indication of the order of appearance of radioactive carbon in these compounds, and it was from an analysis of this sort that we were able to deduce relationships between the various compounds. In much the same way that we deduced the

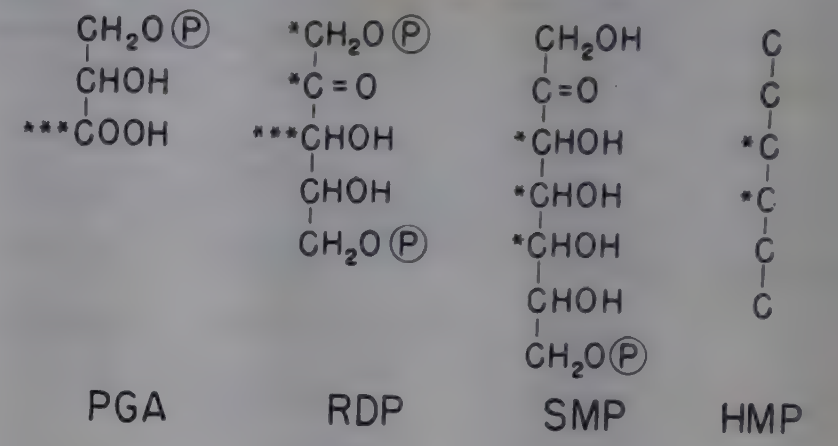



FIG. 8.

relation between the three-carbon PGA and the six-carbon hexoses we were able to deduce the relationship between the five- and seven-, the six- and three-carbon compounds that are shown here. Now actually, it is quite clear at a glance, that there is no simple structural relationship between the five- and the seven- and the other two, nothing at least, as simple as the one between the three-carbon PGA and the six-carbon hexose which appears by simply joining two together. There is no sequence in the $C_5$ or $C_7$ which could be considered as simply the intact $C_3$ or the intact $C_6$, respectively. It wasn't until we realized that the $C_6$ might have more than one origin that we were able to deduce a possible route for its formation. This is shown in figure 9. By taking two carbons off the top of the $C_7$ and adding

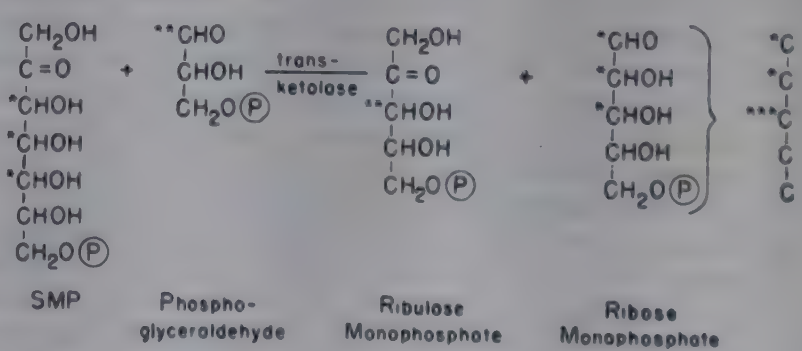



FIG. 9.

them on to a three-carbon piece labeled as is phosphoglyceraldehyde, we would get two five-carbon pieces, one ribulose and one ribose, and their label would be thus distributed. The average of them would be the actual one found. This, then, gave us a clue as to the origin of the ribose and ribulose phosphates which we were finding, namely, by a transketolase reaction of the sedoceptulose phosphate with the triose phosphate to give the two pentose phosphates. These can be interconverted by suitable isomerization. Thus, we have the pentoses formed from heptose and triose.

We know how the hexose is formed, from two trioses. The question then remains; where does the heptose come from? And here, again, a similar detailed and careful analysis of the carbon distribution as a function of time within the heptose molecule was made, and this was a long term job. It finally led to the realization that the heptose must have been made by the combination of a four-carbon with a three-carbon piece. And the question arose then; where does the properly labeled four-carbon piece come from? It could only come by splitting the $C_6$ (hexose) into a $C_4$ and a $C_2$. Figure 10

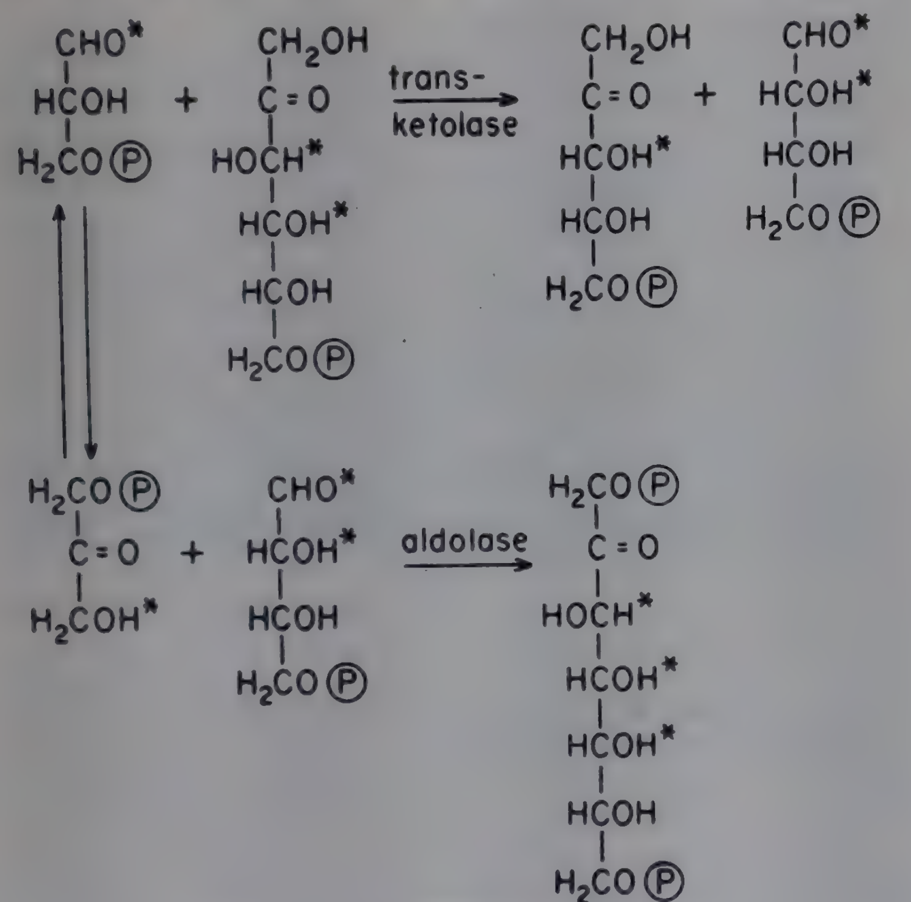



FIG. 10.

shows how this scheme was arrived at. Here are shown the two trioses, which as you know can make a hexose; reacting one triose with a hexose to form a pentose and under the influence of transketolase a tetrose; the tetrose then reacting with another triose, under the influence of the enzyme aldolase, to form the heptose, and giving the proper distribution of carbon. So much, then, for the relation between the various sugars, that is, the triose, the tetrose, the pentose, the hexose and the heptose. We have relationships between all of them and formed them all from PGA, but not any of them yet have been marked (identified) as the precursor which can accept the $CO_2$.

*Identification of $CO_2$ acceptor*

The clue to this was obtained from an entirely different kind of experiment. It was obtained not from an experiment on the rate of appearance of radiocarbon, such as the one I have just described, but from an experiment in which the level of the various compounds was measured the actual total amount of each compound in the plant was measured and then determining the way in which that total amount changed, under changing conditions; For example, the amount of pentose and triose and PGA (that is, the acid from this) and a variety of other things, was determined in a steady state. Then we turned the light off to see what would happen to the various compounds. The results are shown in figure 11. It is

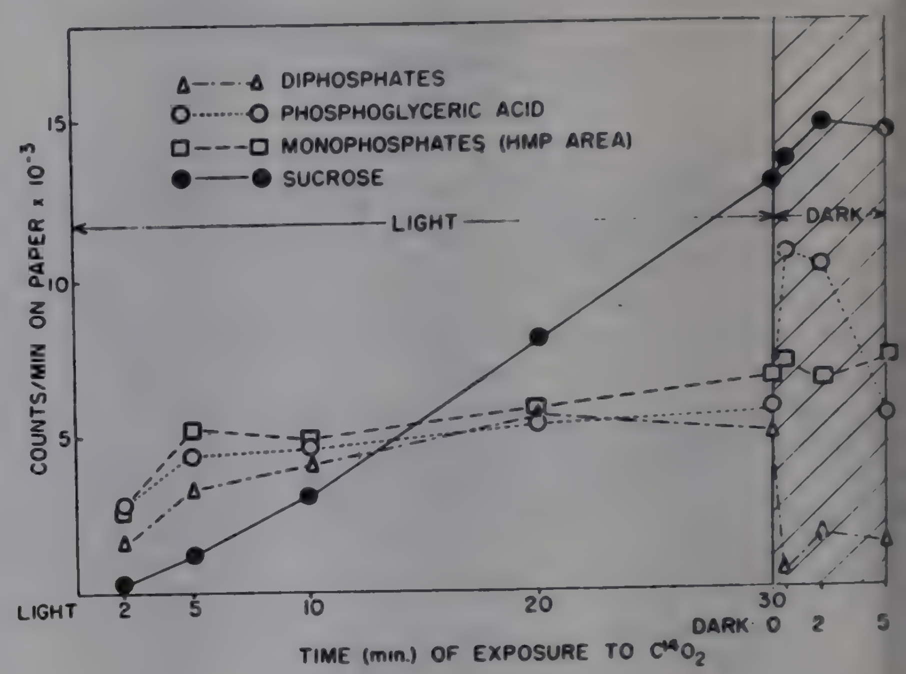



FIG. 11.

clear here that in about five minutes enough carbon has passed into the plant to saturate certain of the compounds (PGA, RuDP, hexose monophosphates) which are therefore functioning in some sort of cyclic operation, and bring them up to the specific activity of the carbon dioxide entering the plant. The sucrose, which is a storage product, is, of course, not saturated; it keeps getting more and more radioactivity. Immediately the light is turned off, a very interesting phenomenon occurs. The ribulose diphosphate, the pentose phosphate, drops precipitously, whereas the PGA increases very sharply. This immediately puts the finger on the RuDP as the immediate precursor to the PGA and suggests a cyclic system such as is shown in figure 12 as a possible scheme for the relationship between the RuDP, PGA, triose phosphate and other sugars. Here it is the RuDP which, upon carboxylation with $CO_2$, gives PGA; we will write the details of that reaction later on. The PGA is reduced by reducing power (hydrogen) which is made by the photolysis of water, to triose phosphate (at the sugar level), the triose phosphate then undergoes a series of rearrangements, such as the ones described earlier, through the hexose, pentose and heptose, back again to the ribulose; this is all at the sugar level of oxidation and requires very little energy for its operation. We thus are able

to regenerate ribulose from triose, and it is clear that if we turn out the light, thus stopping the supply of reducing agent, the PGA should build up if $CO_2$ is present and RuDP will drop. This is precisely what we saw. In fact, it is the reason for drawing this particular scheme.

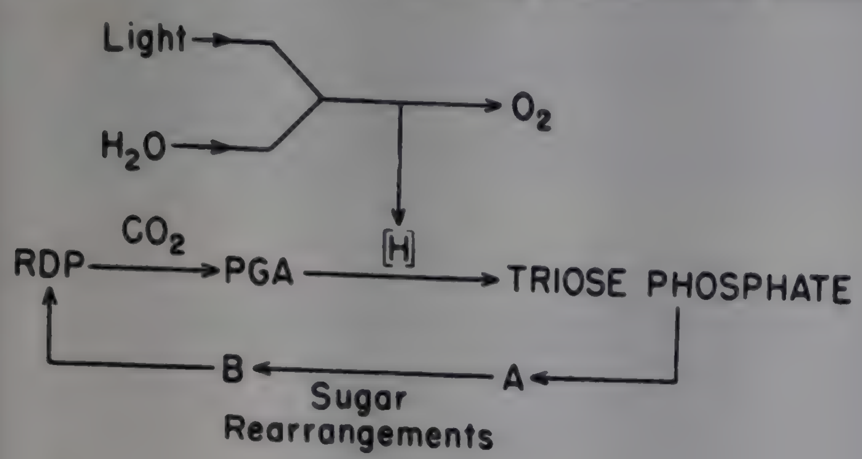
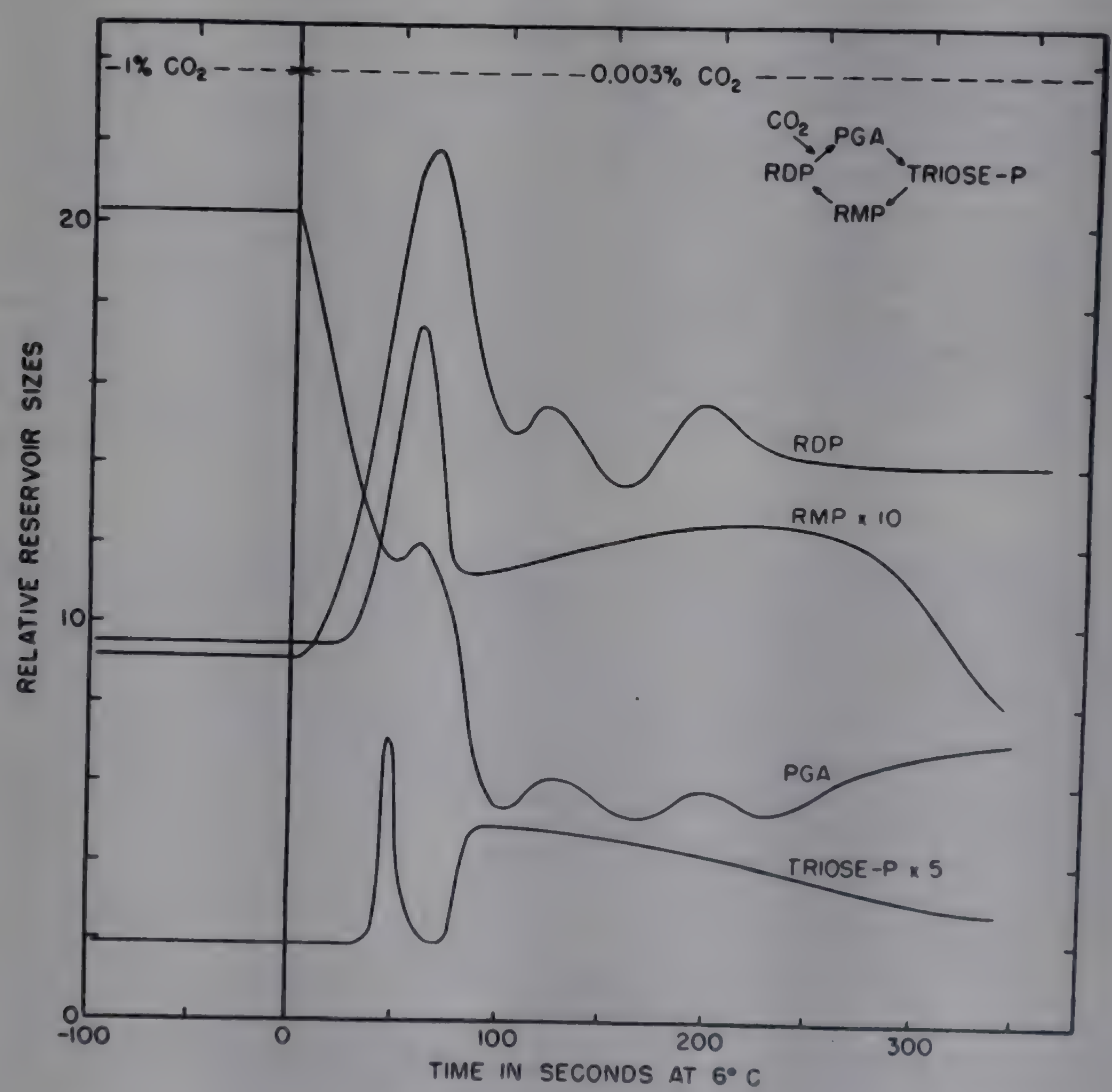



FIG. 12.

There is another deduction which can be made now from this scheme, before the experiment is done, and that is what would happen if the $CO_2$ pressure were suddenly changed. Again, you can see, it is easy to predict that if we suddenly reduce the amount of $CO_2$ present, keeping the light constant (in other words, block the carboxylation reaction) we should build up RuDP and lose PGA. And, indeed, that is what happens and figure 13 shows this result. Here, again, is shown the cyclic scheme of figure 12 together with the sequence of events in its operation. At the time when the $CO_2$ drops from 1 % to 0.003 %, the first thing to rise is the RuDP, the next thing is RMP (ribulose monophosphate) and the last thing to rise is triose phosphate. The first thing to fall on the forward pulse is the triose phosphate; the next thing to fall is the RMP, and the last thing to fall is the RuDP. Thus we have a pretty good confirmation for the operation of such a cyclic feedback system.

## THE CYCLE

Figure 14 shows the whole scheme put together with the details of the structural formulas and enzymes involved. Carbon dioxide enters *via* the formation of PGA by a carboxylation reaction on RuDP. This has been a postulated reaction required by the mechanics and kinetics of the system as we have so far described them. It has not until now been described as an isolated enzyme as has each of the remaining reactions in the cycle. These are : triose phosphate dehydrogenase, giving triose phosphate; the triose then undergoes the various transformations (*via* isomerase) to the ketose, which, with aldolase, makes the fructose diphosphate;




TRANSIENTS IN THE REGENERATIVE CYCLE

FIG. 13.

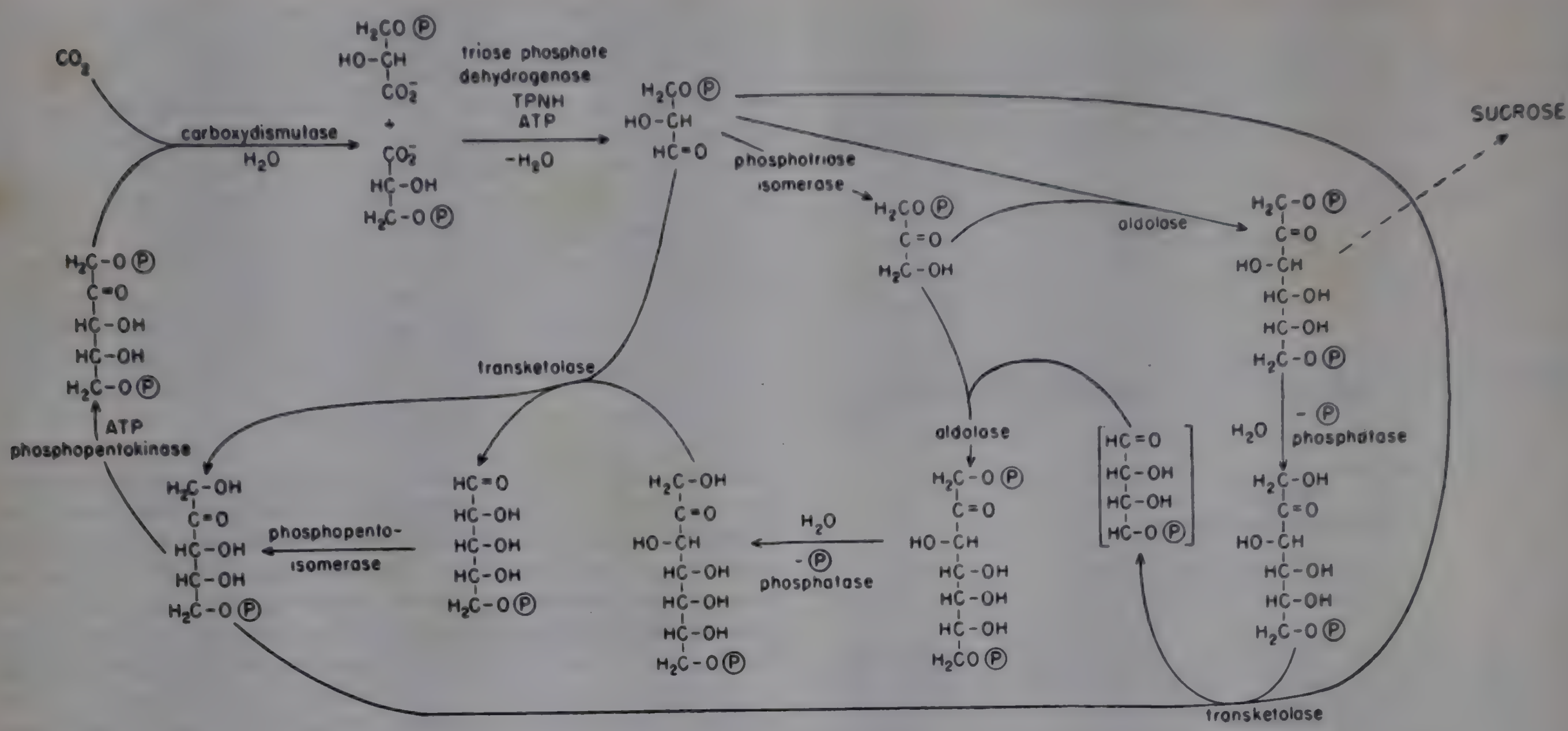



Fig. 14.

with phosphatase to fructose monophosphate, then with transketolase to give the tetrose phosphate, which, again with aldolase, gives the heptose diphosphate; phosphatase to the heptose monophosphate, transketolase again to give both pentose phosphates; ribose phosphate *via* isomerase to give ribulose monophosphate, followed by a phosphorylation, using adenosine triphosphate (ATP) and phosphopentokinase to produce, finally, ribulose diphosphate. Now each of these enzymes from PGA through all of these transformations has been prepared and described. Each of these reactions has been carried out independently in solution, except for the first (or last) one involving $CO_2$.

### *Carboxydismutase*

About a year ago now, directed by our tracer studies, we sought and found a cell-free preparation from both algae and from other green plants which was capable of catalyzing the production of PGA specifically from ribulose diphosphate (RuDP) and sodium bicarbonate. The RuDP used in these experiments was isolated by chromatography from green plant extracts. The technique was to expose the RuDP and the enzyme preparation to $NaH^{14}CO_3$ and show that carboxyl-labeled PGA was formed (figure 15). The trace of malic, citric, aspartic acid, and alanine formed indicates the presence

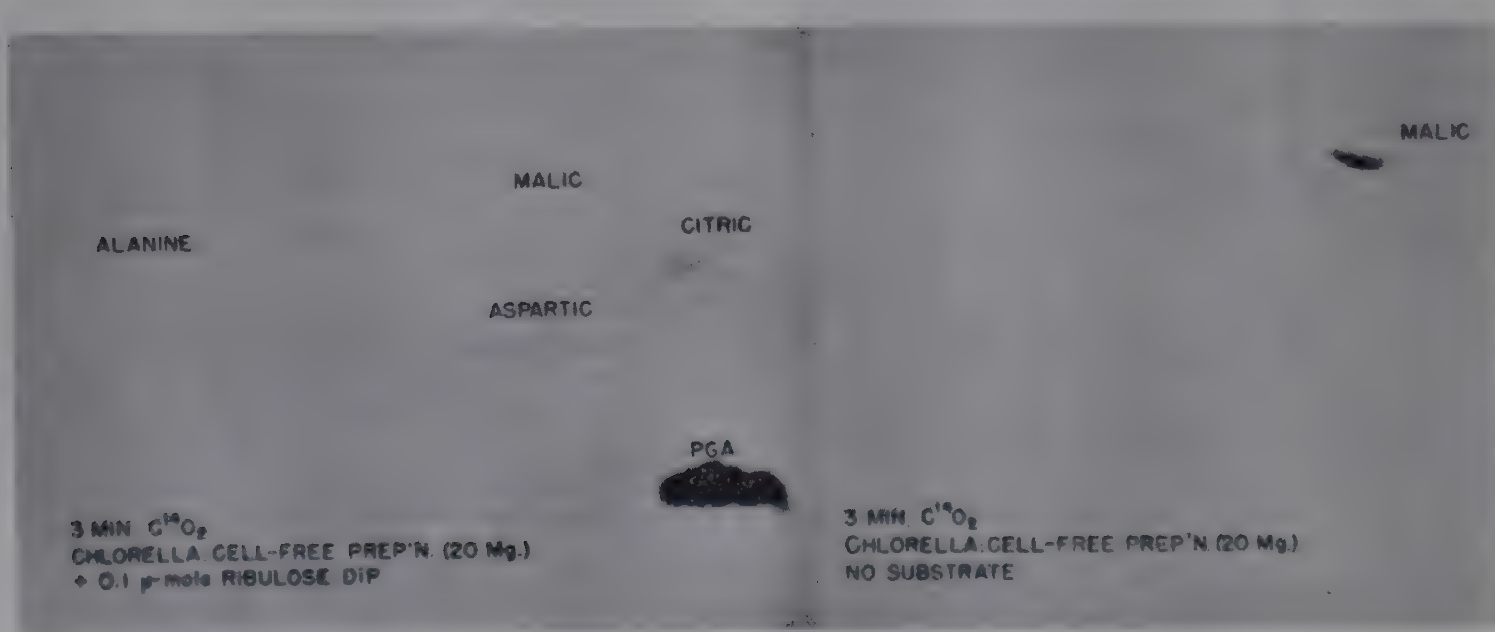



Fig. 15.

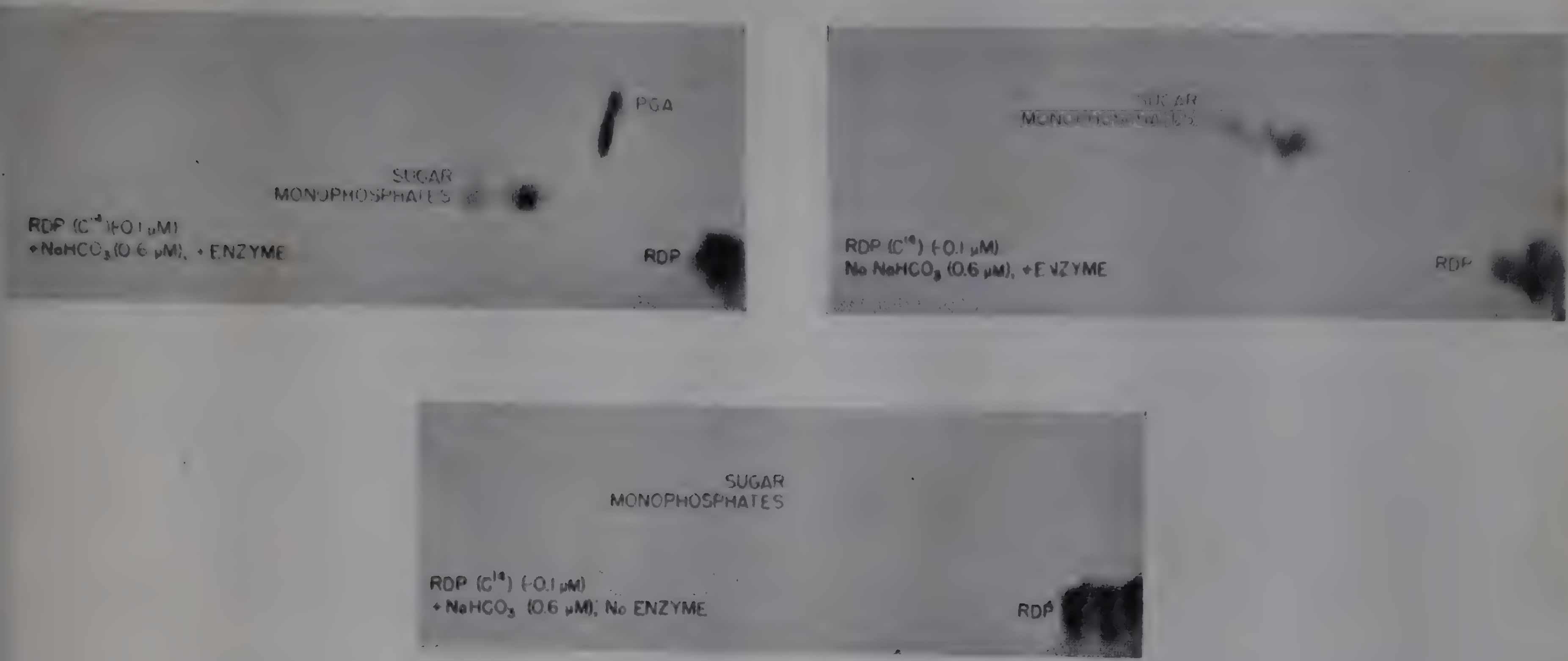


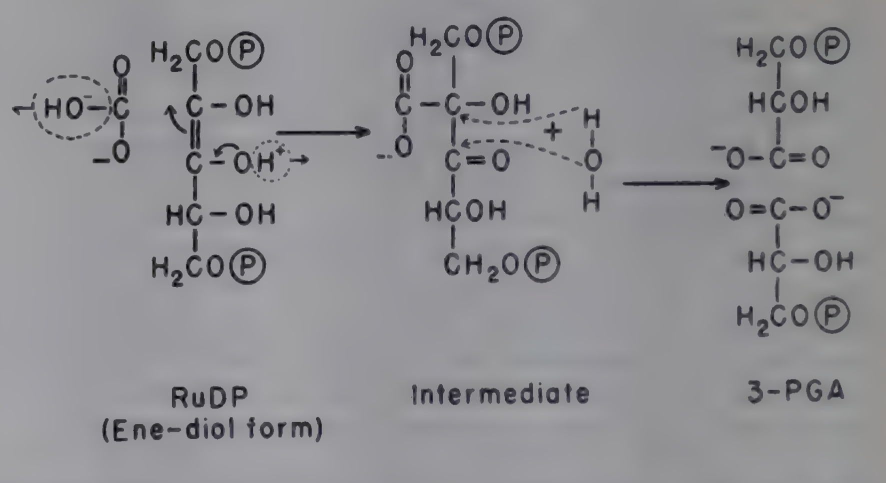

FIG. 16.

of some Krebs cycle enzymes in the preparation as well, which could carry some of the PGA initially formed on to other things. Indeed, upon longer (> three minutes) exposure to these crude preparations, much of the PGA was converted. The formation of a little labeled malic acid in the absence of substrate (RuDP) indicates the presence of pyruvic acid and malic enzyme.

Since in this experiment the tracer was in the $CO_2$ and not in the RuDP it did not give direct information about the fate of the five carbon atoms of ribulose. It was therefore necessary to do the experiment with labeled RuDP and unlabeled $CO_2$. This was not very satisfactory in the first instance when using the crude preparation. Although labeled PGA was formed, a good many other labeled compounds were formed as well because of the presence in the preparation of enzymes which could act on ribulose diphosphate and things formed from it; in particular there was present a phosphatase which permitted the formation of ribulose-5-phosphate. This, in the presence of transketolase, aldolase (and possibly transaldolase) would rapidly find its way into hexose, heptose and triose, the last of which might have given rise to some PGA by oxidation. While attempts to bypass this difficulty by inhibiting the initial phosphatase reaction on RuDP were partially successful because of the insensitivity of the

$$RuDP \xrightarrow{HCO_3^-} PGA$$

system to $F^-$ they were not conclusive (16). It was therefore necessary to proceed with the attempt to free the preparation from any and all other enzymes capable of acting upon RuDP, but the one(s) required for the PGA forming reaction (from $CO_2$). This was accomplished first from neutral extracts of New Zealand spinach (*Tetragonia expansa*) and later from extracts of sonically ruptured algae. The enzyme appears in the protein fraction, salted out of neutral extracts, between approximately 0.3 and 0.4 of saturation with $(NH_4)_2SO_4$. The results of an early experiment with such a preparation acting on labeled RuDP are shown in figure 16 (1). Here the fate of the ribulose carbon is clearly in PGA when both enzyme and $NaHCO_3$ are present. There appears to be some sugar monophosphate present in all of the




experiments, partly due to its presence in the original RuDP sample and possibly partly due to the presence of some residual phosphatase. Later experiments have given preparations which convert essentially all of the ribulose carbon into PGA and nothing else.

It thus appears that the original formulation of the reaction is at least a likely one (*).

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(*) A possibility remains that in the light reductive, fission between $C_3$ and $C_3$ of the carboxylated pentose (enzyme-bound) might take place as an alternative to the known hydrolytic cleavage shown above, and give rise to only one molecule of PGA and one molecule of phosphoglyceraldehyde. At present the evidence seems not to favor this.

Because the carboxylation reaction takes place at the expense of the oxidation of the $C_3$ of the ribulose to the carboxyl level, the name 'carboxydismutase' suggests itself as uniquely descriptive. It is interesting to note that the enzyme is not readily demonstrated in animal tissues (rat liver) and that it can be obtained from spinach in association with the highly organized intact chloroplasts (2) from which it is extremely easily separated. It does not appear to be especially sensitive to versene, *o*-phenanthroline or cyanide, but it is sensitive to *p*-chloromercuribenzoate which inhibition is reversed by cysteine (16,1).

### *Chemical requirements to run the cycle*

Now to return to the scheme of things again. We now have the cycle in its details (figure 14) and we know now precisely what reagents are required to make the cycle turn. Let us have a look at the reagent requirements to keep this cycle running and to see if we can determine something about the energy requirements for it. You can see here that what is required for the reduction of a PGA molecule to a triose is one molecule of triphosphopyridine nucleotide (TPNH) and one molecule of adenosine triphosphate (ATP). There is no further energy requirement in any of the sugar rearrangements, until we get to the point of ribulose-5-phosphate, where we require again another molecule of ATP to make the RuDP. A calculation of what is required per $CO_2$ molecule entering will show that the net requirement for the production of triose phosphate, that is, the net reduction of one molecule of $CO_2$ to the carbohydrate level is four equivalents of reducing agent, that is, four electrons, and three molecules of ATP. Thus we have the complete energy requirement, at least as far as the $CO_2$ is concerned, established. For each molecule of $CO_2$ that goes to carbohydrate we have to supply four electrons (four equivalents of reducing power) and three molecules of ATP. All of this must be made ultimately by the light, by the conversion of the electromagnetic energy in some way. You will notice that in this requirement for reducing carbon there is no particular requirement for a photochemical reaction other than the production of the two reagents. If we could supply those two things from some other source than the photochemical reaction, we should be able to make this whole sequence of operations function. We have reason to believe that this is indeed being done by the use of the required collection of enzymes. This has been accomplished by Racker (17). But a suitable situation exists in nature also. The situation is such that we must have simultaneously a high level of this particular reducing agent, which we now know can be triphosphopyridine nucleotide (TPN) and ATP at the same time and the same place.

### *Running the cycle without light*

There is one system in nature, aside from the green plants, in which that situation occurs. At least we know about this one; it is one of the photosynthetic purple bacteria which doesn't make oxygen, but does reduce carbon dioxide with molecular hydrogen. Figures 17 and 18 (3) will show that it is possible to have the reduction of $CO_2$ take place either through the agency of light or through the agency of a chemical oxidation system. The organism is the purple bacteria, *Rhodopseudomonas capsulatus*. The initial slope corresponds to the reduction of carbon dioxide in the light; this is using hydrogen as the reducing agent, but light is required.

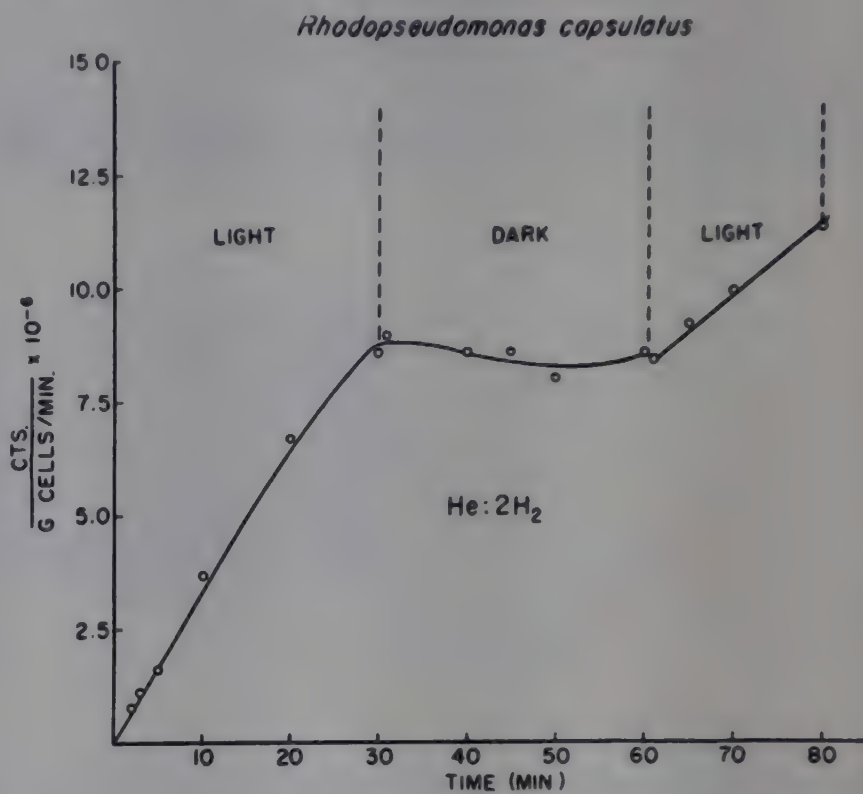



FIG. 17.

As soon as the light is turned off, the carbon dioxide stops being reduced. Figure 18 shows the same organism. This is a dark fixation. Here it is exposed only to helium and hydrogen, and there is an initial fixation which immediately saturates and then stops; but then when oxygen is admitted to the system, the fixation again

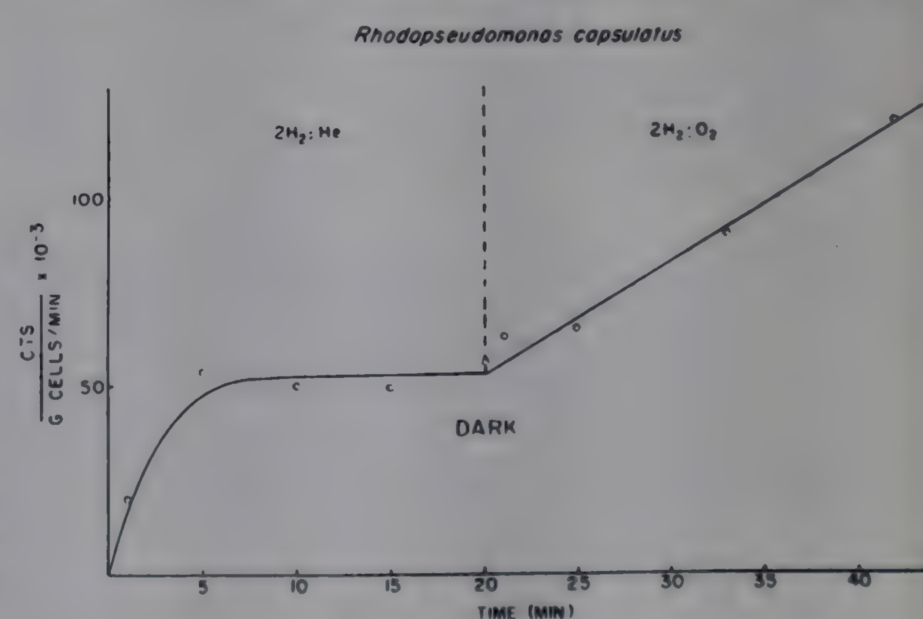



FIG. 18.

continues in the same way as it does with light. The intermediates in the dark are very much the same. The hydrogen is presumably there to produce the reducing power that is needed. The oxygen is required to oxidize some of that hydrogen to make ATP, and the two together, then, can make the carbon dioxide cycle function.

This suggests that a prime function of the light requirement when the high reducing potential of hydrogen is the reducing agent is to supply the oxidizing agent necessary for the production of the required ATP.

*Quantum requirements*

In order to estimate what a minimum quantum requirement for photosynthesis might be on the basis of the information we have so far accumulated about the detailed chemistry of the process, at least one assumption is necessary relating to the mode of interaction of electromagnetic radiation and matter. It is that a single quantum can excite not more than a single electron. Another assumption about the behavior of the excited electron is required, namely, that it does not by some chemical (or physical) dismutation process give rise to more than one equivalent of reducing power at the potential of TPNH. And if that is the case, then one can, just from looking at the requirements we mentioned a few moments ago, predict what the minimum quantum requirement for such an operation, not counting the efficiency of the oxygen evolution and of the system, would be. We need the four electrons for the reduction, and we need three molecules of ATP. Now we already know something about the various ways in which ATP can be produced. We know, for example, that by the transfer of two electrons from TPNH to an atom of oxygen (one-half mole of oxygen) we can produce approximately two or three molecules of ATP. Therefore, one can suppose, on this basis, that the minimum quantum requirement, when all of the energy for the operation of this cycle comes from light and only from light ultimately must be six, that is, four electrons for the reduction and two more for the three molecules of ATP that are required. However, it should be possible to find conditions under which the quantum requirement for the reduction of $CO_2$ and the evolution of oxygen would be as little as four, provided there was some other source besides the light for the three molecules of ATP. The quantum requirement determination was quite accidental. We happened to have the apparatus set up in which one could measure directly, without any ambiguity, the production of oxygen by a direct measurement of some unique quality of the oxygen, not merely a gas pressure, and this quality happened to be the paramagnetism. Also we could measure directly the amount of carbon dioxide absorbed, by measuring some property of the $CO_2$ in the gas phase, not merely its pressure, and this happened to be its infrared spectrum. Between the two of these, by direct measurements, then, of the gases evolved and absorbed under the influence of light in a system in which the light absorbed could be directly determined without too much complication in terms of scattering, a very flat system, we were able to measure the quantum requirement for oxygen production under a variety of conditions. Figure 19 shows the type of equipment which was used. Figure 20 shows the kind of data that you get when the output of the two analyzers is fed into a multipoint recorder. The lines correspond to the $CO_2$ and $O_2$ partial pressures. From the volume of the system we can calculate exactly how many moles of each are being absorbed, or emitted, per minute. From the light absorbed we can calculate the quantum requirement, and this result is shown in figure 21. Here the solid points give the apparent quantum requirement for net oxygen evolution without any correction whatever. This is assuming nothing; it

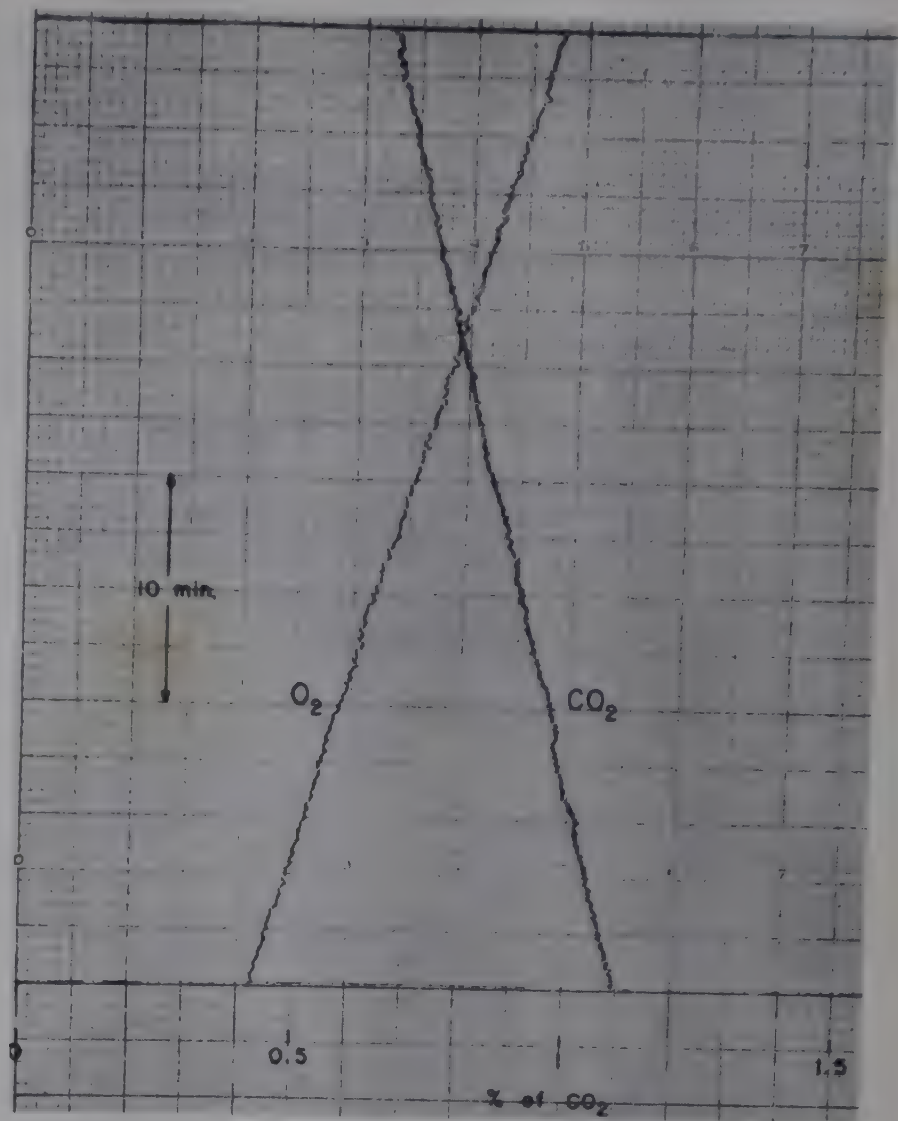



FIG. 20.

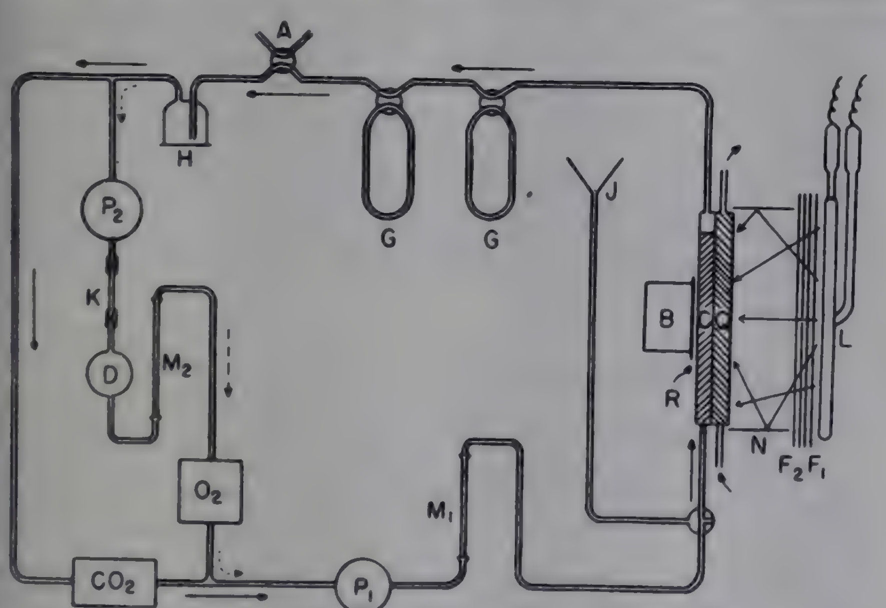



FIG. 19. — Schematic diagram of apparatus for measuring quantum requirements of photosynthesis. C : algae vessel; B : bolometer; Q : water jacket; N : reflecting tube; $O_2$ : oxygen analyzer; $CO_2$ : carbon dioxide analyzer; A : stopcock; K : capillary; $P_2$ : pump; D : bulb; G : tubes containing $N_2$; H : rubber membrane; J : inlet for introduction of algae; F : filters; M : flow meters; R : opal glass.

is simply the number of molecules of oxygen evolved divided by the number of quanta of light absorbed in the same time. And you see that at very high light intensity ($q$ is the number of quanta absorbed per second) the quantum requirement drops down to something less than 8 (about 7, or 7½ or thereabouts). The P/R scale is the ratio of the rate of photosynthesis to

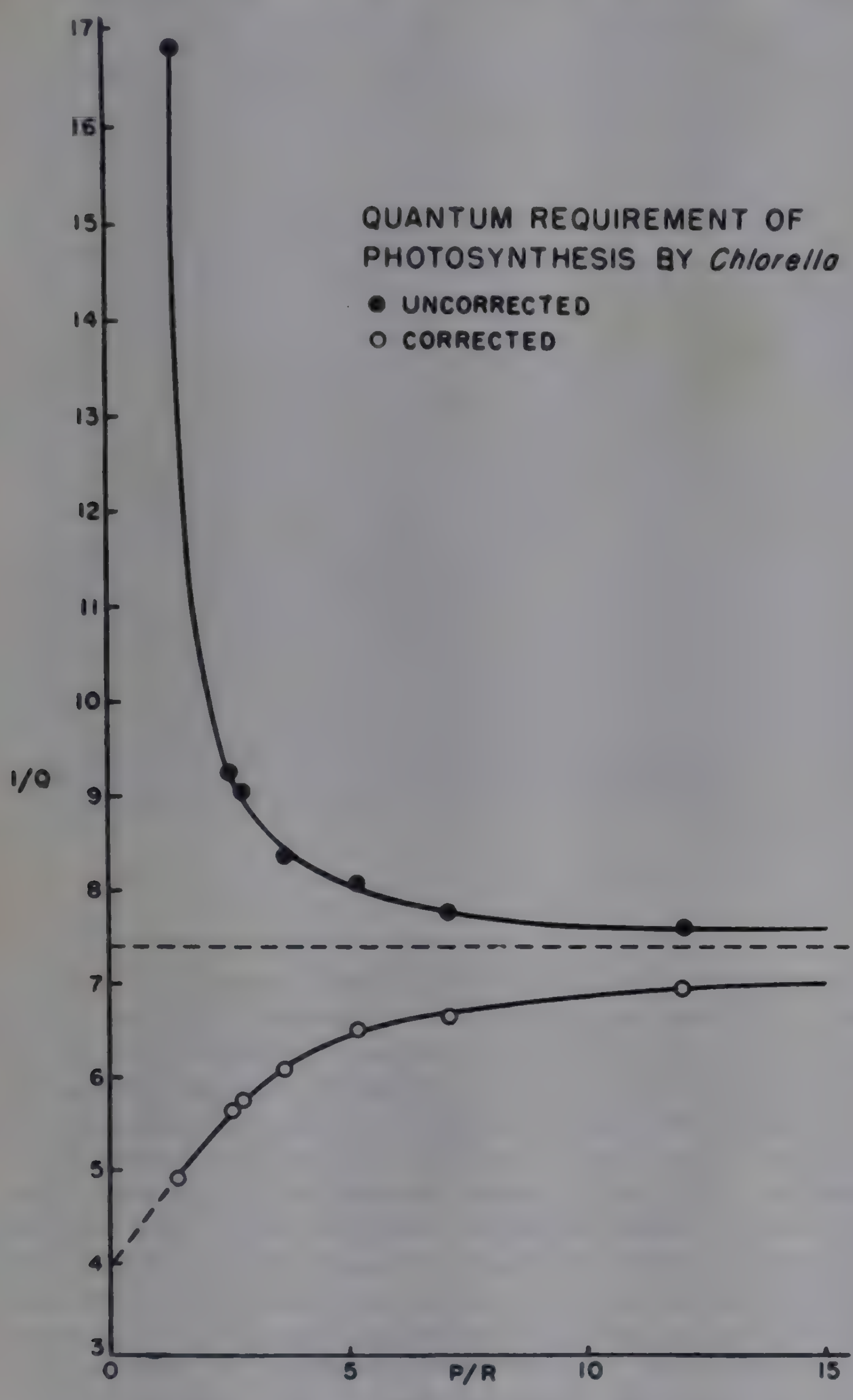



FIG. 21.

the rate of respiration in the immediate dark period following that photosynthetic measurement. In the high intensity range the rate of photosynthesis was ten-twelve times that of the reverse reaction, that is, the absorption of oxygen in the dark period immediately following. If we assume, and we have every reason to assume this is so, that the oxygen absorption in the light period just preceeding is going on at very nearly the same rate as it does when you turn off the light and this rate of re-absorption is added to the net evolution of oxygen, then the quantum requirement falls to that corresponding to the open points. As the amount of respiration with respect to the photosynthesis gets larger and larger, the quantum requirement gets smaller and smaller. You see it extrapolates at low levels of photosynthesis to about four (*). Under these conditions, the light is not called upon, or at least the electrons generated by the quanta are not called upon, to produce ATP; they can be used exclusively for the reduction process, and so the quantum requirement may be as low as four. But if the rate of reduction becomes so great that the rate of generation of ATP outside of the chloroplasts is not fast enough to keep up — or at least the diffusion rate is not fast enough and ATP must be generated at the site of reduction — then we must use some of those photo-produced electrons to make ATP and the quantum yield will fall, or the quantum requirement will rise, to something approaching seven.

This seemed like a very useful and good confirmation of the general notion that we have so far presented. I would like to tell you how the light makes the electrons and how the electrons can be converted into ATP when it is necessary (but unfortunately I don't know yet). However, we do have some very stimulating indications and I will try to tell you about them.

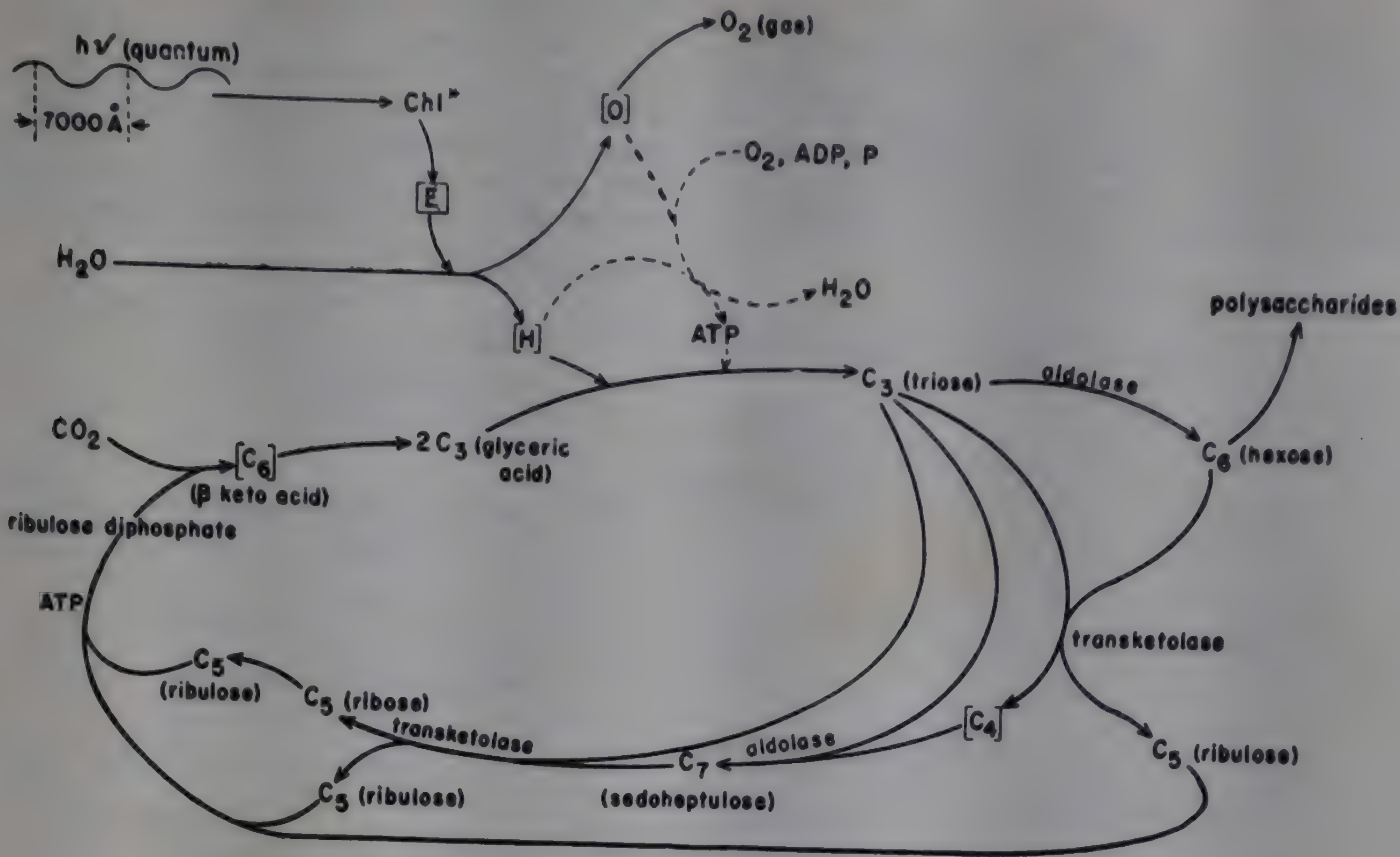
## QUANTUM CONVERSION

So far we have been talking only about the reduction of carbon. And because this seems to be quite a separate system from the oxygen evolution reaction, it would appear that we wouldn't expect to learn a great deal about the photoproduction of the electrons and the ATP from studying the carbon reduction. But it so happens that there is, and obviously there must be, a connection between the two, and that connection is fairly close. By suitable observations we were able to see at least one point at which the carbon reduction cycle makes contact directly with the photochemical apparatus. This is shown in figure 22. Here is the cycle again. The quantum is first absorbed by chlorophyll and converted into something making a reducing agent [H] and some oxidizing agent [O]; the reducing agent can reduce the glyceric acid to triose. Some of the reducing agent must be used to make ATP, with oxygen or the intermediates on the way to oxygen, because that is necessary for the cycle to run; this is what we have already talked about. What we are going to talk about now is this point of contact [H], between the photochemical apparatus and the carbon cycle and what information we can learn about the quantum conversion part from studying this.

### *Light inhibition of TCA cycle incorporation*

The experiment that was done was one in which a steady state was examined and the changes induced by a sudden change of conditions were observed. Figure 23 shows this. Here we have the same type of experiment as before but we are looking at different substances now. Focus your attention particularly on glutamic

(*) This value of four as the quantum requirement at low photosynthetic rates is in no way comparable to the values between three and four reported by Warburg and his associates at very high P/R ratios (>20) (5).




PROPOSED CYCLE FOR CARBON REDUCTION
IN PHOTOSYNTHESIS

FIG. 22.

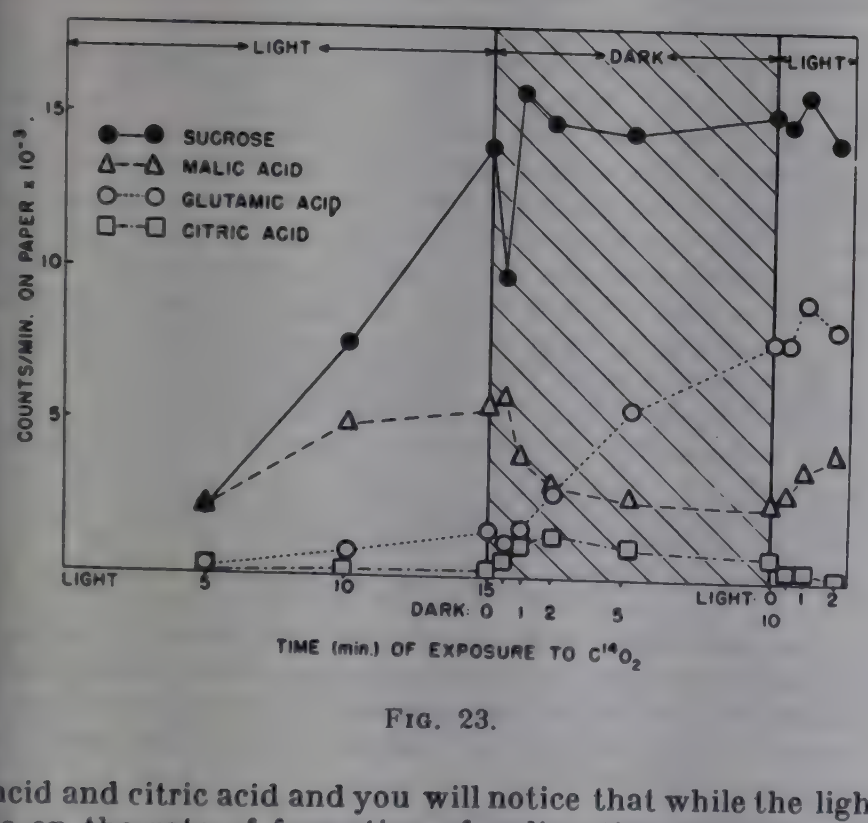



FIG. 23.

acid and citric acid and you will notice that while the light is on the rate of formation of radioactive glutamic acid and radioactive citric acid is quite low. But immediately the light is turned off, the rate of formation of radioactive glutamic acid is increased manyfold; the rate of formation of radioactive citric acid also increases manyfold. Now glutamic and citric acids are two compounds very closely related to the respiratory cycle known as the Krebs cycle, and figure 24 will describe in schematic terms the experimental facts we have just seen. Here is shown the photosynthesis cycle with which you are

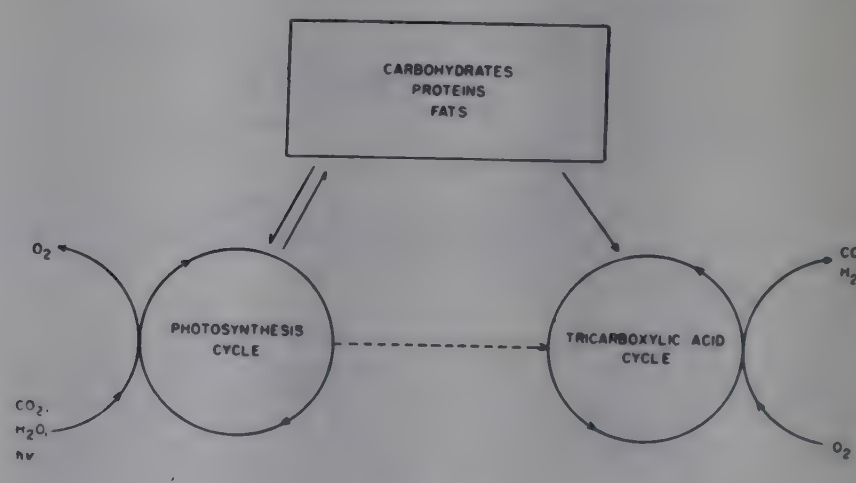



FIG. 24.

already familiar. Here, also, is the Krebs or tricarboxylic acid cycle, the details of which are shown in figure 25. The glutamic acid and citric acid are in or related to the Krebs cycle.

The photosynthetic cycle you already have seen, and it does not contain either glutamic or citric, but it does, as you know, form PGA and the sugars. Eventually these direct products of the photosynthesis cycle have

to get back into the tricarboxylic acid cycle. That is the major route in the light. But immediately the light is turned off there is a direct connection between the two cycles, apparently opened up, which allows the PGA to go over directly into the compounds of the tricarboxylic acid cycle. Figures 25, 26, 27 show the details of that mechanism. Figure 25 shows the tricarboxylic acid cycle and how the carbon can get into that cycle *via* acetyl-coenzyme A, condensing with oxalacetic acid to citric acid, which is one of the compounds we talked about, going around this route and leading over to glutamic acid. The question is : how does glyceric acid get to acetyl-coenzyme A ?

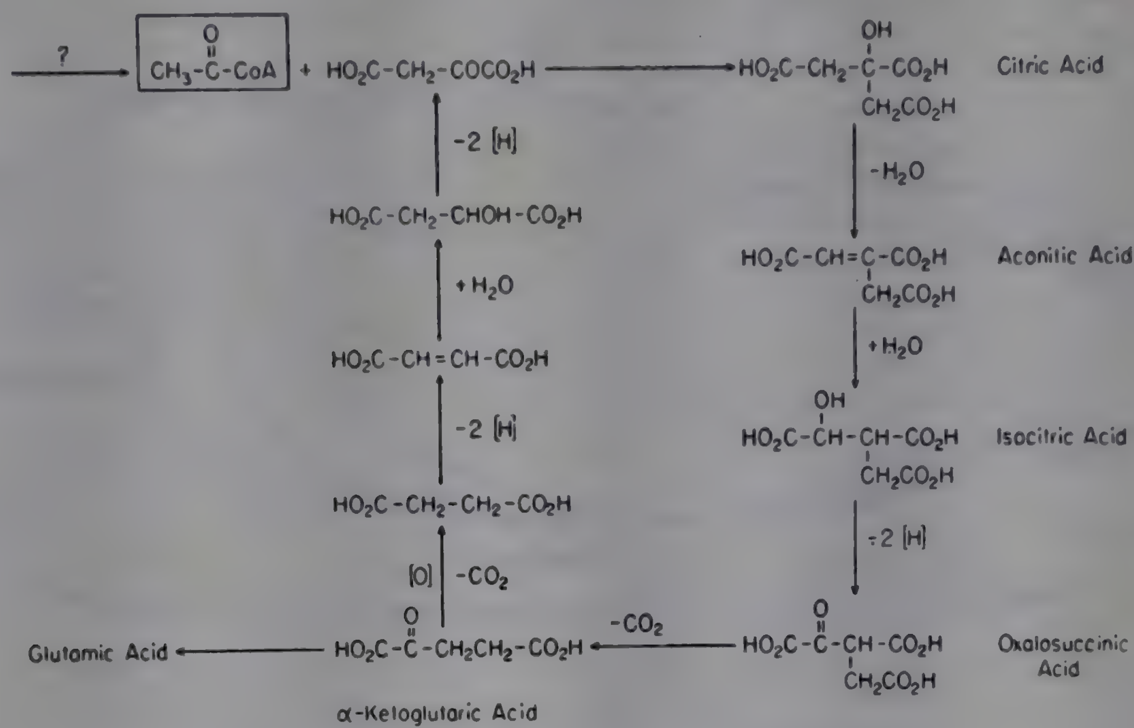



FIG. 25. — Tricarboxylic acid cycle.

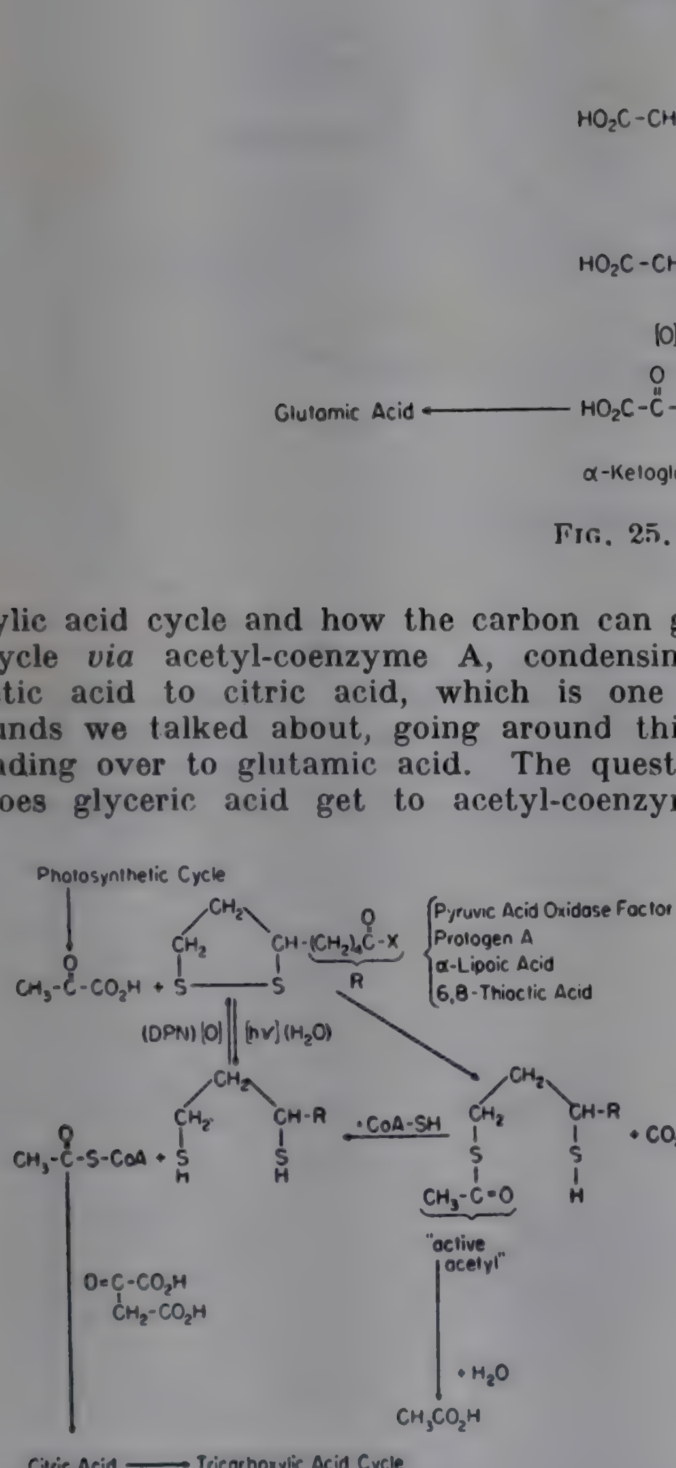


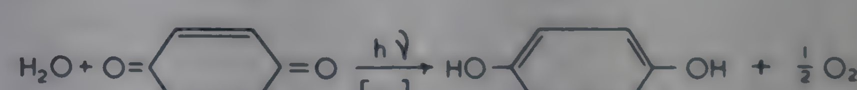

FIG. 26.

This must happen rapidly only in the dark, not very rapidly in the light. Fortunately we have some idea how acetyl-CoA may be formed from glyceric acid and figure 26 shows this. The glyceric acid is dephosphorylated to form pyruvic acid ; the pyruvic acid then reacts with an enzyme system of which thioctic acid is a coenzyme to form acetyl-thioctic acid and carbon dioxide. The acetyl-thioctic acid then undergoes a thiol ester interchange with CoA to form reduced thioctic acid and acetyl-CoA, which then goes on into the citric acid cycle in the way shown in figure 27 (6).

Now, how does light affect this ? This reaction (fig. 26) is the door for the entrance of carbon into the tricarboxylic acid cycle, and if somehow we close this door by removing or reducing the level of the disulfide, we can reduce the rate of appearance of radioactive carbon from the photosynthetic cycle into the citric acid cycle. This, then, suggests that the light shifts the equilibrium from the disulfide to the dithiol form by inducing reaction with something other than pyruvic acid, probably ultimately water. In the dark, oxidation brings it back again to the disulfide and the thing starts working again. This system is like a valve which is closed by the light and which controls the flow of carbon from the photosynthetic cycle directly into the tricarboxylic acid cycle. It suggests further that the disulfide may be closely allied to, if not exactly, the electron acceptor from the photochemical act. Actually, a number of experiments have been performed that indicate that this may be so.

### *Thioctic acid effects*

For some time now it has been possible to produce oxygen from water by illumination of grana, chloroplasts, or whole algae in the presence of some suitable electron acceptor (oxidizing agent ) other than $CO_2$, for example, quinone (Hill reaction) :



where [C] is a chlorophyll-containing structure, having some minimum size and degree of organization. If the


FIG. 27. — Some relationships between the photosynthesis cycle and respiratory cycles; (1) Krebs cycle and (2) pentose cycle. ——— reductive (photosynthesis) pathways; - - - - oxidative pathways.

thioctic acid (or some relative of it) is indeed as closely associated with the photochemical part as above suggested it might be expected that its addition to a suitably limited system would increase the quantum yield for oxygen production. This has indeed been observed (7). Although you could increase the quantum yield of oxygen production by adding thioctic acid it could only be done under conditions under which the electron acceptor was rate limiting.

Figure 28 (7) shows the increase of the quantum yield by adding thioctic acid to a system which is producing oxygen; quinone is the electron acceptor. And it works only under conditions where the electron acceptor is rate limiting, so the thioctic acid can be the electron acceptor but does not have to be the acceptor of the oxygen of the water molecule. In fact, the differential increase in rate per mole of added thioctic acid may be ten times that for quinone, indicating its much greater efficiency for this process in spite of the fact that thermodynamically it is much the more difficult to reduce.

While this indicates that thioctic acid can and does function between the light activation and the reduction of quinone it does not answer the question of whether or not there is anything between the light activation and the reduction of thioctic acid. Thioctic acid acceleration experiments with flashing light (8) (approx. 200 microsecond flashes) in which the dark time between flashes was sufficiently long so that no reaction not directly involving the photoactivated state could be rate limiting, failed to give an unequivocal answer, partly because a high enough intensity per flash to saturate was not reached. They did indicate, however, that with

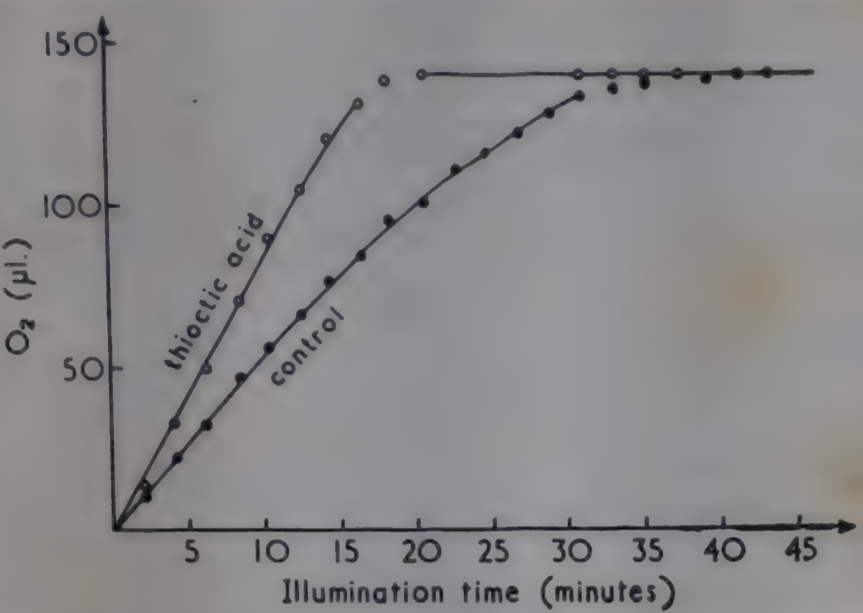



FIG. 28. — The thioctic acid effect on the rate of oxygen evolution by illuminated algae in the presence of quinone (see reference 7).

the concentrations of thioctic acid available — as determined by its biological activity for the growth of propionate-inhibited *S. faecalis* (9) — the lifetime of the electronically excited condition in the natural functioning chlorophyll-containing structure (C) should be quite long, longer than anything observed for chlorophyll in molecular solutions. It should be specifically stated that this requirement obtained only if the thioctic acid had to function directly in the mechanism of oxygen evolution as well as in the reduction part (10). However, the original notion (11) of its function involved only its reduction to dithiol, *i.e.* as electron acceptor. This kind of separation of function is given further support by the apparent insensitivity to mercury poisoning of the Hill reaction with quinone (12) as well as some other considerations. If therefore we give up the idea that the thioctic acid has to accept both the hydrogen and oxygen in the splitting of the water molecule and has to accept only the hydrogen, that is, only the electron, with something else accepting the oxygen or, if you like, taking the electron away from the oxygen, then the situation is relieved and we can retain the thioctic acid as the acceptor of excited electrons only. Something else has to be recognized as the electron donor, or, in other language, the acceptor of the positive hole left by the removal of the excited electron.

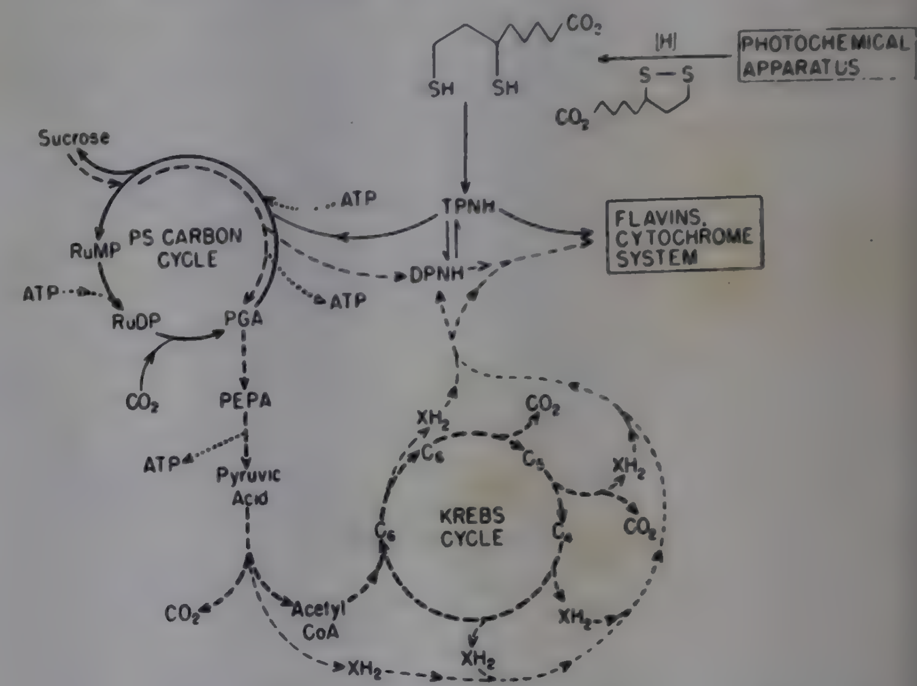



FIG. 29*a*. — Diagram of the suggested nature of the photochemical apparatus and its relation to other functions. - - - - oxidative, or respiratory, pathways; ——— reductive, or photosynthetic, pathways.

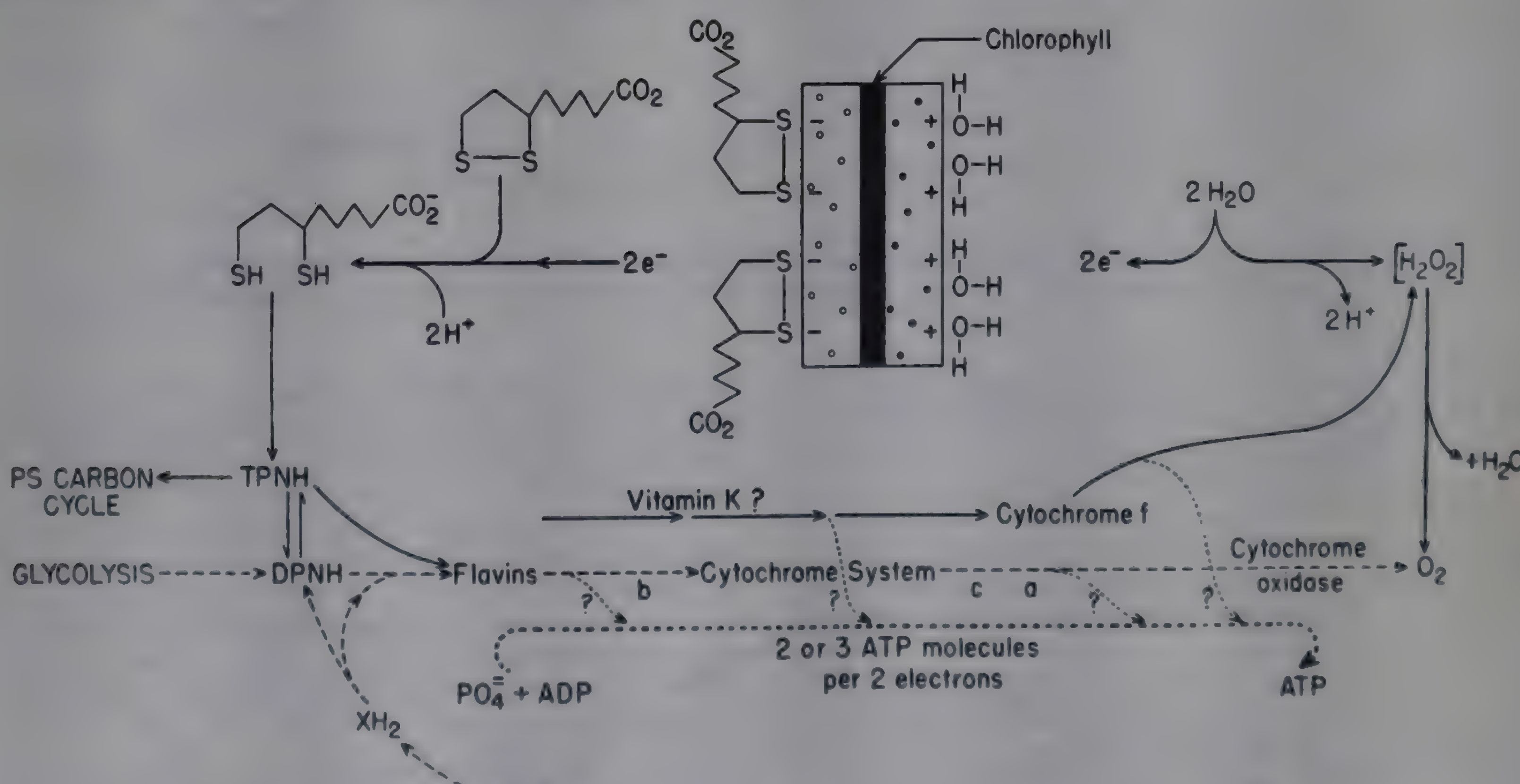



FIG. 29*b*. — Diagram of the suggested nature of the photochemical apparatus and its relation to other functions. - - - - oxidative, or respiratory, pathways; ——— reductive, or photosynthetic, pathways.

With this we arrive at a proposal which not only has precedent in physics and physical chemistry and resolves most, if not all, of the apparent difficulties we have so far recounted, as well as much information not mentioned here (6), but also provides a function for the type of microstructure now becoming apparent (laminations on a macromolecular level) within the subunits (grana) of the chloroplasts (13). It involves the basic notion that the chlorophyll functions photochemically as an organized, oriented aggregate, and not as individual molecules resembling in this one respect the 'photosynthetic unit' (14, 15) and that the absorption of light in this aggregate raises an electron from a molecular level to a conduction level. Figure 29 (6) contains a schematic representation of this notion, together with its relationship to the photosynthesis cycle, the Krebs cycle and oxygen evolution and the phosphorylation mechanism (ATP producing system). The electrons and holes photocreated are immediately separated to opposite sides of a laminated structure by the presence of a built-in field, possibly such as exists at an *n-p* junction. The 'electrode' reactions taking place are written out on either side of the triple layer diagram (figure 21).

One of the major problems to which this suggestion provides an answer is this question of the lifetime of the excited state. You probably have heard many arguments leading to the final conclusion that because of the efficiency and kinetics with which the photosynthetic process proceeds, either the excited state must last for a very long time, or else the converting factor — the energy acceptor — must be present in concentrations equal to or as great as that of the chlorophyll. There is no single molecular species which is present in that amount except water or its close relatives (possibly carotenoid alcohols). So here we are now putting the water molecule at one end of a photobattery, if you like (fig. 29), with the disulfide at the other. The conducting electrons created in the chlorophyll layer migrate to one side of the double layer, the holes are immediately trapped on the other side by electron donation from the water molecules, which are present in large amounts, so the back reaction cannot go on. We have then long-lived electrons which can wait at the opposite side of the layer for the sulfur compound to go through its cycle several times and pick them all up. This is the suggestion as it now stands and which is consistent with most of the information we have today.

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## The cytochrome components and chlorophyll in relation to the carbon cycle of Calvin

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(*Received* 3 *October* 1955)

Before I open this discussion perhaps I may make a few comments and raise a question for your consideration.

First, I am sure that you would all like to join with me in congratulating Dr. Calvin on his brilliant success in the elucidation of the metabolism of $CO_2$; culminating, may we say, in the prediction and in the subsequent discovery of the enzyme system which he has called carboxydismutase. We have, I think, been shown clearly the energy requirements of this carbon cycle.

Many of us, now, will be asking for more details as to how the light energy is converted into a chemical form. The two chemical forms required by the carbon cycle are reducing power and phosphorylation energy. The photochemical production of a reducing system *in vitro* was studied by Hill (1), Holt and French (2), Warburg and Lüttgens (3) and others. The *in vitro* photochemical process of phosphorylation was first accomplished by Vishniac and Ochoa (4) with a mixed chloroplast and mitochondrial system; more recently photochemical phosphorylation has been found by Arnon, Allen and Whatley (5, 6) to occur with washed suspensions of chloroplasts, and the same result was shown to be obtained with cell free extracts of the photosynthetic bacterium *Rhodospirillum* sp. by Frenkel (7). The energy required for esterification of the inorganic phosphate is at present considered to be derived from the reoxidation of a substance reduced by the action of light.

In any chemical system, if a substance becomes reduced, a corresponding amount of another compound must be oxidised.

In respiration the oxidations and reductions take place with the chemical gradient. In a photochemical system reductions and oxidations can take place against the chemical gradient (that is in relation to an equilibrium as determined by a 'dark' process). Such reactions result in the conversion of light energy into chemical energy. When the chemical forms of energy become applied to the carbon cycle, carbon dioxide would be assimilated by 'dark' processes which will have to run with the chemical gradient as in respiration. The critical steps, now, are concerned with the fate of the oxidised product resulting from the action of light. In the green plant the final product is molecular oxygen derived from the 'oxidation' of, for example, water; in the photosynthetic bacteria the final product arises by oxidation of a hydrogen donor supplied by the environment (8). In addition, however, it seems that part of the oxidised product at some stage or another is utilised (9) for the reoxidation of part of the (photochemically) reduced product to supply the energy for a phosphorylation process. In short, chloroplast preparations would seem to have an analogue of oxidative phosphorylation, as indicated by Arnon and coworkers, which is driven by light and not by respiration.

Some time ago Scarisbrick and I (10, 11) began a study of the cytochrome components in leaves. At the same time Bhagvat (12) was able to show that in the chlorophyll-free parts of plants the particulate fractions derived from the cytoplasm contained the cytochrome system as defined by Keilin (13) and by Keilin and Hartree (14). This system is now recognised to be characteristic of mitochondria obtained from a wide variety of sources. The green tissue, however, contained cytochrome *f* which is different from cytochrome *c*, though closely related to it.

Scarisbrick and I could find no cytochrome oxidase, specific for cytochrome *f*, which would catalyse its oxidation by molecular oxygen. This negative result by itself means little but I want to emphasise it now and to attempt to define differences in the haem-proteins present in particles from the white and the green tissues of plants.

Davenport (15) studied the cytochrome in plastids from developing etiolated leaves. In barley the cytochrome components are in the chloroplasts before the appearance of chlorophyll. In these yellow plastids (which, by the way, are similar in size to normal green plastids) cytochrome *f* and cytochrome $b_6$ (16) are present. A little cytochrome *a*, but very little, is present in the preparations that we examined (figure 1, B). We do not know yet whether this small amount of *a* is all due to contamination of the chloroplast preparations by particles normally characteristic of the colourless tissue.

It appears that in the green plastids there is a development of the $b_6$ and *f* components. Instead, perhaps, of the *a* components there is the large amount of the light absorbing pigment chlorophyll, there being from 500 to 1000 times more of this than of the cytochrome components : *f* and $b_6$ (figure 1, C). That the cytochrome components are functional in relation to the photo-

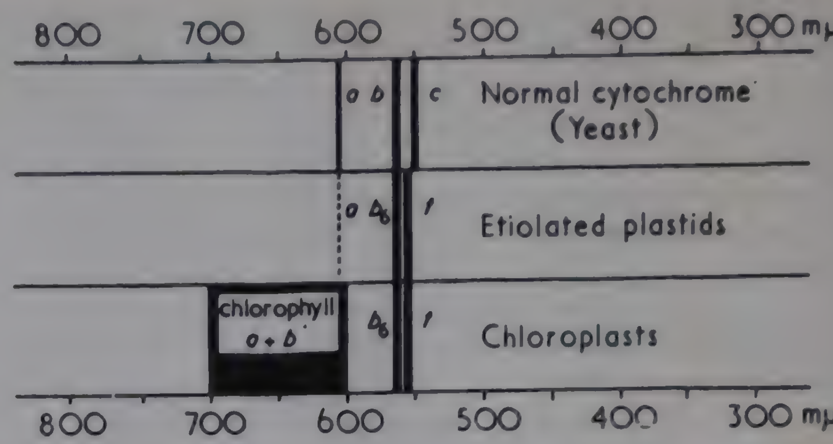


FIG. 1. — Diagram showing the positions of the α-bands of cytochrome components characteristic of mitochondria (A) and plastids (B and C). In C the absorption of the chlorophylls in chloroplasts is shown as a wide band to indicate the much higher concentration as compared with the cytochrome components.

chemical system of the chloroplasts would be indicated from the conclusions of Duysens (17) and of Lundegårdh (18); they were able to show that the oxidation of a cytochrome component (probably cytochrome *f*) occurs under the influence of light. Thus it may be that the cytochrome system in the illuminated chloroplasts is behaving like that in the mitochondria during respiration. In darkness, owing to the absence of a cytochrome oxidase for cytochrome *f*, there would be no rapid transfer of H or of electrons through the cytochrome system to molecular oxygen.

In the active plastid material, as soon as it is illuminated, there appears to be a transfer of electrons in two directions both against and with the chemical gradient. It is this property, perhaps, of the chloroplast system which has so often defeated experiments to set up individual partial reactions *in vitro*.

In the chloroplasts the chlorophylls and the carotinoid pigments are firmly bound to the structure, this is also true of the cytochrome components $b_6$ and *f*. This situation seems also to be the case for the cytochrome components in the mitochondria. We are led to ask how far structural relationships are important in a chemical sense for the function of these cytoplasmic particles. Dr. Calvin has suggested that the chlorophyll may be concerned in the separation of two types of substance represented by analogy with the *n*- and *p*-types of semiconductor in a rectifying barrier photocell. This rectification idea may well be profitable, for indeed the distance between layers of staining material shown by the electron microscope technique with chloroplast sections is of the order of 100 Å (19, 20). This is similar to the range of action, for example, in a CdO/selenium boundary (21). The chloroplast is not likely to be crystalline to the extent of allowing us to express the properties which refer to semiconductors in terms of crystal lattice replacements by atoms with excess or deficiency in valence. The production of 'holes' and 'electrons' by the action of light was considered in relation to photosynthesis by Katz (22) in order to explain the formation of the oxidising and reducing agents as a result of energy transfer in the chloroplast substance. It would be desirable, however, to have a more chemical interpretation of the action at the *n*-/*p*-junction in a barrier photocell (23). Hence it seems that while the

problem referring to photosynthesis may be queried in terms of the physical structure, the problem of the contact layer in a barrier photocell may be queried in more purely chemical terms.

The important biological implication seems to be that the plastids and the mitochondria show similarities indicated both by the structure and by the presence of cytochrome components associated with insoluble material. The light absorbing pigments in chloroplasts can also be regarded as part of the structure. The question that I would now like to raise is how far the chloroplasts or the photosynthetic chromophores can be said to resemble mitochondria, modified by the presence of chlorophyll which replaces the '*a* part' of a cytochrome system.

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# Photosynthesis by isolated chloroplasts


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(*Received* 9 *September* 1955)

The old concept of chloroplasts as the site of the complete photosynthetic process in green cells was largely abandoned in the last two decades (see review, 1). The substitute view, which gained ascendancy on the strength of new experimental evidence, regarded chloroplasts as structures 'much simpler than required for photosynthesis' (2) and ascribed to them *in vivo* only the limited functions of photolysis of water and the transfer of electrons and protons from water to such acceptors as pyridine nucleotides (3). Other reactions in photosynthesis were, in this view, linked to the reoxidation of the photo-chemically reduced pyridine nucleotide by enzyme systems situated outside the chloroplasts, as for instance in the generation, by oxidative phosphorylation of adenosine triphosphate (ATP) by mitochondria, and in $CO_2$ fixation by reductive carboxylations of the type exemplified by the malic enzyme reaction (3).

Recent experiments in our laboratory (4, 5, 1) have provided a new basis for the old concept of the localization of all photosynthetic reactions in the chloroplasts. Complete photosynthesis, *i.e.* the reduction of carbon dioxide to the level of carbohydrates with a simultaneous evolution of oxygen, has been carried out *in vitro* with isolated chloroplasts unaided by other enzyme systems. Although it seems premature as yet to equate it with photosynthesis *in vivo*, photosynthesis by chloroplasts *in vitro* offers special opportunities for the study of the photosynthetic process in a cell-free system without the attendant complications of extraneous metabolic reactions. Extracellular photosynthesis by chloroplasts proceeds in the absence of respiration, oxidative phosphorylation or other cellular processes requiring the consumption of molecular oxygen.

The purpose of this brief communication is to discuss the present status of our work. In our first experiments (4, 5, 1) extracellular photosynthesis was attainable only with intact chloroplasts. Photosynthesis has now been attained with broken chloroplasts, showing that the structural integrity of the chloroplasts is not essential. A historical survey of extracellular photosynthesis has been presented elsewhere (1) and an extensive discussion of the literature on phosphorus metabolism in photosynthesis is being reserved for separate treatment (6).

*Reactions of isolated chloroplasts*

Isolated chloroplasts, whether whole or broken, have long been known to have a capacity to evolve oxygen when illuminated in the presence of an artificial electron

(*) Aided by grants from the National Institutes of Health and the Office of Naval Research.

acceptor (Hill reaction) in accordance with equation (*1*).

$$\text{Light} + A + H_2O \longrightarrow H_2A + \tfrac{1}{2}\,O_2 \qquad (1)$$

in which A represents an electron or hydrogen acceptor other than carbon dioxide. Reaction (*1*) has until recently represented the only known photochemical activity of isolated chloroplasts and has, for this reason, sometimes been called « the chloroplast reaction » (Hill, 7; Whittingham, 8). However, it has recently been established that isolated chloroplasts are also capable of carrying out two additional photochemical reactions : photosynthetic phosphorylation (4, 9), represented by equation (*2*), and also, as shown by equation (*3*), the reduction of $CO_2$ to the level of carbohydrate with an accompanying evolution of $O_2$ (5).

$$\text{Light} + \text{ADP}\,(^*) + P_i(^*) \longrightarrow \text{ATP}\,(^*) \qquad (2)$$

$$\text{Light} + H_2O + CO_2 \longrightarrow (CH_2O) + O_2 \qquad (3)$$

### *Photosynthetic phosphorylation*

In the light, but not in the dark, isolated chloroplasts vigorously synthesize ATP from inorganic phosphate and AMP (adenosine-5′-phosphate) or ADP. In our earlier experiments (4, 9) the structural integrity of the chloroplasts was considered essential for photosynthetic phosphorylation. It has now been found that on destroying the structure of the whole chloroplasts by treatment with water, their capacity for photosynthetic phosphorylation was unimpaired when the requisite cofactors, flavin mononucleotide (FMN), vitamin K and ascorbate, were added under anaerobic conditions. The response of broken chloroplasts to the addition of the cofactors of photosynthetic phosphorylation (table I) was similar to that of whole chloroplasts (10, 11). The enzymes involved in photosynthetic phosphorylation are apparently unaffected by the disruption of chloroplast structure through the water treatment.

Photosynthetic phosphorylation is independent of $CO_2$ fixation and of the dark reactions of respiration. This conclusion, based earlier on experiments in which $CO_2$ was excluded from the system (4, 9), is now reinforced by the finding that broken chloroplasts can, without the aid of other enzyme systems, synthesize ATP but cannot fix $CO_2$. Furthermore, a number of substances inhibit $CO_2$ fixation without affecting photosynthetic phosphorylation.

A point of special interest is whether the conversion of light into the pyrophosphate bond energy of ATP proceeds through oxidative phosphorylation involving mitochondria, as previously shown in model systems (12), or through pathways peculiar to photosynthesis. The evidence summarized below favors the latter conclusion.

(*a*) Photosynthetic phosphorylation is an anaerobic process (10, 11, 1, also compare 13); molecular oxygen is neither produced nor consumed during the phosphorylation. This was first shown by manometric measurements (4). The lack of any oxygen evolution was also confirmed by the very sensitive luminous bacteria method for the detection of molecular oxygen (5). Equation (*2*) for photosynthetic phosphorylation is visualized as the sum of equations (*4*) and (*5*) :

$$\text{Light} + H_2O \longrightarrow 2\,[H] + [O] \qquad (4)$$

$$2\,[H] + [O] + \text{ADP} + P_i \longrightarrow H_2O + \text{ATP} \qquad (5)$$

TABLE I

*Comparison of effects of cofactors on photosynthetic phosphorylation by whole and broken chloroplasts*

| Additions (μ-moles) | | | $P_i$ esterified (μ-moles) | |
|---|---|---|---|---|
| Menadione | FMN | | Whole Chloroplasts | Broken Chloroplasts |
| 0 | 0.1 | Complete | 7.0 | 10.5 |
| 0.003 | 0.1 | Complete | 6.8 | 9.5 |
| 0.03 | 0.01 | Complete | 11.7 | 11.5 |
| 0.03 | 0.1 | Complete | 14.3 | 17.2 |
| 0.3 | 0 | Complete | 16.0 | 18.2 |
| 0.03 | 0.1 | Complete | 12.6 | 20.0 |
| 0.03 | 0.1 | Mg omitted | 0.34 | 5.7 |
| 0.03 | 0.1 | Ascorbate omitted | 2.7 | 1.9 |

The complete reaction mixture contained 40 μ-moles Tris [tris-(hydroxymethyl)aminomethane] buffer, pH 7.2, 20 μ-moles inorganic phosphate ($P_i$), 20 μ-moles adenylic acid, 10 μ-moles Na ascorbate, menadione (2-methyl-1,4-naphthoquinone or vit. $K_3$) and flavin mononucleotide (FMN) as specified, in a total volume of 3.0 ml. Phosphorylation was measured as previously described (9, 10). Whole chloroplasts were prepared in 0.35 M NaCl, and the reaction mixture made to volume with this salt solution. Broken chloroplasts were prepared by suspending washed whole chloroplasts in distilled water, and the reaction mixture made to volume with water.

In reaction (*4*) light energy is consumed in the photo-decomposition of water. The oxygen and hydrogen atoms in brackets represent an oxidized and a reduced product of this photolysis (not molecular oxygen or hydrogen). In reaction (*5*) these photo-decomposition products recombine to reconstitute water and the energy liberated thereby is stored in the pyrophosphate bonds of ATP.

(*b*) ATP synthesis is not enhanced by the addition of pyridine nucleotides to either whole or broken chloroplasts. It is unlikely that this can be explained by the presence of an adequate supply of bound pyridine nucleotides since water treatment results in the loss of most of the nucleotide present in the whole chloroplast without affecting ATP synthesis in the light. As will be discussed later, the addition of these coenzymes greatly stimulated $CO_2$ fixation by broken chloroplasts; hence it seems they are concerned in this aspect of photosynthesis rather than ATP synthesis.

(*c*) Photosynthetic phosphorylation does not depend on the presence of mitochondria. It is accomplished by washed chloroplasts without the aid of other enzyme systems. The addition to chloroplasts of all the other cytoplasmic particles (including mitochondria) from

(*) $P_i$ represents orthophosphate ; ADP, adenosine diphosphate ; ATP, adenosine triphosphate.

spinach leaves gave no increase in ATP synthesis. Particles resembling mitochondria, which carry out oxidative phosphorylation in the dark but no photosynthetic phosphorylation in the light, have been isolated from spinach leaf homogenates after removal of the whole chloroplasts (see table II). On the other hand,

TABLE II

*Photosynthetic and oxidative phosphorylation by particulate fractions from leaves*

| | Photosynthetic phosphorylation (gas phase nitrogen) | Oxidative phosphorylation (gas phase air) | |
|---|---|---|---|
| | $P_i$ esterified (μ-moles) | $P_i$ esterified (μ-moles) | $O_2$ absorbed (μ-atoms) |
| Whole chloroplasts . . . . | 17.6 | 0.2 | 0.5 |
| Remaining particles . . . | 3.2 | 6.2 | 3.0 |
| All particles . . . . . . . | 11.6 | 1.2 | 1.0 |

Whole chloroplasts were prepared by centrifugation of leaf homogenate for 7 min. at 1000 *g*. Remaining particles were prepared by centrifugation at 18 000 *g*, after removal of whole chloroplasts. All particles were prepared by centrifugation of leaf homogenate for 15 min. at 18 000 *g*. Leaf homogenate was prepared by grinding in a citrate-sorbitol-borate buffer (21). For measurement of photosynthetic phosphorylation the particles were suspended in 0.35 M NaCl and anaerobic esterification of inorganic phosphate in the light was measured as described previously (9, 10). For measurement of oxidative phosphorylation the particles were suspended in the citrate-sorbitol-borate buffer and the esterification of inorganic phosphate and the oxygen uptake measured aerobically in the dark in a system containing 40 μ-moles Tris buffer, 20 μ-moles phosphate ($P_i$) containing $^{32}P$ (9), 20 μ-moles adenylate, 10 μ-moles $MgSO_4$, 1 % glucose, and hexokinase. The volume was made to 3 ml. with the citrate-sorbitol-borate buffer. Particles containing 0.5 mg. chlorophyll were used in each experiment.

washed whole chloroplasts or broken chloroplasts, which carry out photosynthetic phosphorylation in the light without the addition of any enzyme system or substrate, show no endogenous oxygen uptake and do not carry out any oxidative phosphorylation in the dark or in the light when supplied with Krebs cycle intermediates or reduced pyridine nucleotides.

It is concluded from this chain of evidence that in green plants the generation of ATP occurs at two distinct sites : in the mitochondria, adapted to phosphorylation of the oxidative type needed to maintain cellular activity during periods of darkness and in the chloroplasts, adapted to the direct conversion of light energy into pyrophosphate bond energy during photosynthesis.

This conclusion does not rule out the possibility that certain individual steps in the electron transfer during photosynthetic phosphorylation are similar to those in oxidative phosphorylation. The catalysts so far identified for photosynthetic phosphorylation, Mg++, FMN, vitamin K, and ascorbate, are all either known to be or are suspected of being involved in oxidative phosphorylation as well.

### *$CO_2$ fixation*

Isolated whole chloroplasts, unaided by other enzyme systems, were found on exposure to light to fix $CO_2$ with a simultaneous evolution of oxygen (4, 5). $CO_2$ fixation was measured by incorporation of $^{14}CO_2$. The reaction was strictly light-dependent, and proceeded at an almost constant rate for at least an hour (5). There was approximate correspondence between the oxygen evolved and the $CO_2$ fixed, as would be expected for green plant photosynthesis. Both soluble and insoluble products resulted from the fixation of radiocarbon by chloroplasts. The insoluble product was identified as starch, which appeared to be the only insoluble compound formed (5). Among the soluble products the following compounds have so far been identified : phosphate esters of fructose, glucose, ribulose, dihydroxyacetone, and glyceric acid ; glycolic, malic and aspartic acids ; alanine, glycine, and free dihydroxyacetone and glucose.

The ability of chloroplasts to fix $CO_2$ was almost completely lost when their structure was damaged by treatment with water (table III). Their $CO_2$-fixing capacity was partially restored when a clear, straw-colored extract obtained by treating whole chloroplasts with water was added. The addition to the broken chloroplasts of DPN, TPN, or ATP, singly or in combination, failed to restore their capacity for $CO_2$ fixation in the absence of the chloroplast extract, but increased photosynthetic $CO_2$ fixation in the presence of the chloroplast extract. Heating the extract almost completely abolished its effect on $CO_2$ fixation by broken chloroplasts. $CO_2$ was reduced to the level of carbohydrate by the broken chloroplast system. The products,

TABLE III

*Effect of adenosine triphosphate and di- and triphosphopyridine nucleotides (DPN and TPN) on the fixation of $^{14}CO_2$ by broken chloroplasts ($Pl_w$) supplemented with a water extract of chloroplasts (CE)*

| Additions | $Pl_w$ | $Pl_w$ + CE | $Pl_w$ + heated CE |
|---|---|---|---|
| None . . . . . . | 950 | 137 300 | 19 750 |
| ATP . . . . . . | 1300 | 204 950 | 35 400 |
| DPN . . . . . . | 2250 | 347 700 | 20 250 |
| TPN . . . . . . | 1600 | 450 250 | 19 450 |
| ATP + DPN . . | 2000 | 411 950 | 35 550 |
| ATP + TPN . . | 2800 | 659 350 | 29 900 |

$Pl_w$ was prepared by suspending whole chloroplasts, prepared in 0.35 M NaCl in distilled water. CE was prepared by suspending whole chloroplasts in water and discarding the green particles by centrifuging at 20 000 *g* for 10 minutes. For measurement of $CO_2$ fixation, $Pl_w$ containing 0.5 mg. chlorophyll, was added to a solution containing 40 μ-moles Tris buffer, pH 7.2, 10 μ-moles Na ascorbate, 2 μ-moles $MnCl_2$. CE equivalent to chloroplasts containing 2 mg. chlorophyll, 1 μ-mole ATP, and 0.3 μ-mole DPN or TPN were added as specified. Total volume 3 ml. $CO_2$ fixation was measured as previously described (5). Results are expressed in $^{14}CO_2$ fixed (C.P.M.).

shown in figure 1, were similar to those obtained with whole chloroplasts, except that malic and aspartic acids were absent.


**Fig. 1. — Chromatogram showing products of photosynthetic $CO_2$ fixation by broken chloroplasts. Compound directly above area labeled 'monophosphates' has been identified as phosphoglyceric acid. Experimental procedures as given in table III and as previously described (5).**

These results (table III) indicate that treating whole chloroplasts with water removes both cofactors and soluble enzymes which are required for $CO_2$ fixation. The identification of the enzymes contained in the chloroplast extract is now in progress; among those already identified are the glyceraldehyde phosphate dehydrogenases of green leaves (14), 'carboxydismutase' (15), phosphoglyceryl kinase, phosphopentokinase, phosphoglucomutase, menadione reductase, aldolase and phosphorylase. The role of these and other water-soluble enzymes of chloroplasts in $CO_2$ fixation is currently under investigation.

Photosynthetic $CO_2$ fixation by broken chloroplasts supplemented with chloroplast extract, TPN, and ATP equalled or exceeded that obtained with the most active preparations of whole chloroplasts. An additional several-fold increase in the rate of $CO_2$ fixation was obtained by the further addition of any one of a number of compounds, principally phosphorylated sugars (table IV). As seen from the diagrammatic summary in figure 2, the combined effect of the various addenda has resulted in a level of $CO_2$ fixation several times higher than that obtained with whole chloroplasts. This high level of photosynthetic activity by broken chloroplasts is expected to aid materially in the study of the detailed mechanism of extracellular photosynthesis.

The effect of ATP in restoring the capacity for $CO_2$ fixation to broken chloroplasts is in harmony with the conclusion (1, 4) that ATP is a prerequisite for $CO_2$ fixation by chloroplasts. As with whole chloroplasts, phosphorylated sugars were identified among the products of $CO_2$ fixation by broken chloroplasts (figure 1). It seems possible (cf. 3) that pyridine nucleotides ($PN^+$) are reduced by chloroplasts during a photolytic cleavage of water in accordance with equation (*6*) (see below). The

Table IV

*Effect of phosphorylated compounds on photosynthetic carbon dioxide fixation by broken chloroplasts ($P/_w$) (10 micromoles $CO_2$ added)*

| Additions | $CO_2$ fixed ($\mu$-moles) |
|---|---|
| None (*) | 2.0 |
| 3-Phosphoglyceric acid | 3.7 |
| Ribose-5-phosphate | 4.0 |
| Glucose-1-phosphate | 4.4 |
| Glucose-6-phosphate | 3.8 |
| Fructose-6-phosphate | 3.1 |
| Fructose diphosphate | 5.2 |

(*) $P/_w$, CE, ATP, TPN.

3 $\mu$-moles of each phosphorylated compound was added to the reaction mixture. Other experimental conditions as described in table III.

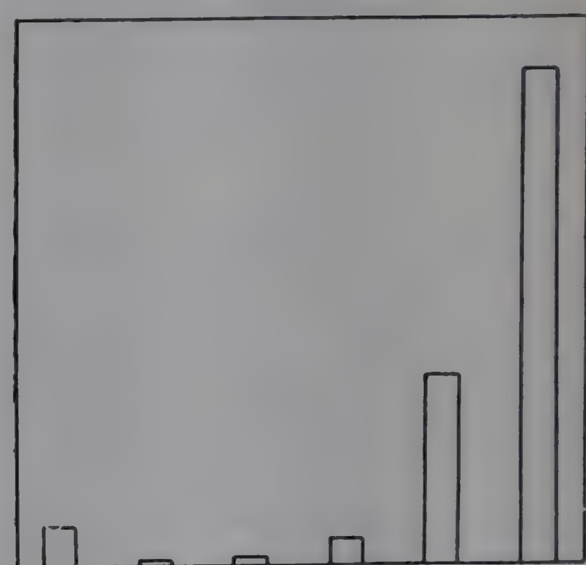


Fig. 2. — Diagrammatic representation of the effects of various addenda on $CO_2$ fixation by broken chloroplasts. Experimental conditions as in tables II, IV and III. *A*, whole chloroplasts; *B*, water-treated chloroplasts; *C*, water-treated chloroplasts + adenosine triphosphate (ATP) + triphosphopyridine nucleotide (TPN); *D*, water-treated chloroplasts + chloroplast extract; *E*, water-treated chloroplasts + chloroplast extract + ATP + TPN; *F*, water-treated chloroplasts + chloroplast extract + ATP + TPN + fructuòse-1,6-diphosphate.

reduced pyridine nucleotides could then serve as electron carriers in the reduction of $CO_2$ according to equation *7*.

$$\text{Light} + H_2O + PN^+ + ATP \rightarrow PNH + H^+ + \tfrac{1}{2}O_2 + P_i + ADP \qquad (6)$$

$$CO_2 + 2\,PNH + 2\,H^+ + n\,ATP \rightarrow (CH_2O) + 2\,PN^+ + n\,ADP + P_i \qquad (7)$$

Like ATP synthesis, the photochemical $CO_2$ fixation by chloroplasts is linked with a photolysis of water. Only in $CO_2$ fixation is the oxygen of water released as a free gas while the hydrogen of water becomes a part of the sugar molecule (equations *6*, *7*, and *3*). In photosynthetic phosphorylation, as previously stated, the photolytic cleavage of water (equation *4*) is followed by its reconstitution (equation *5*), before any evolution of molecular oxygen takes place.

It is envisaged (see also, 16) that ATP participates in the reduction of pyridine nucleotides (equation *6*). In this manner it is possible to retain the concept discussed elsewhere (1) that the key reaction in photosynthesis, the photolysis of water, is accomplished by the energy of one quantum of red light (*ca.* 43 kcal.). The pyrophosphate bonds of ATP could provide the supplementary energy for chloroplast reactions which require an input of more than 43 kcal. Taking the standard oxidation-reduction potential of TPN at pH 7 as —0.32 V, it would require approximately 50 kcal. to give a $TPN_{red}/TPN_{ox}$ ratio of 1.

The reduction of $CO_2$ to the level of carbohydrate (equation *7*) almost certainly involves a reduction of a carboxyl to a carbonyl group. The reducing potential of pyridine nucleotides is insufficient to accomplish this step without a lowering of the potential barrier, which could again be achieved by ATP phosphorylating the carboxyl group prior to its reduction by pyridine nucleotides (17).

*General scheme for extracellular photosynthesis*

In the light of present evidence chloroplasts appear as remarkably complete cytoplasmic structures which contain multienzyme systems divided into three main groups, each controlling an increasingly complex phase of photosynthesis : photolysis of water, photosynthetic phosphorylation, and $CO_2$ fixation. Whole chloroplasts contain all three groups of enzymes. Broken chloroplasts (table III) contain only two ; the group of $CO_2$ fixing enzymes is leached out by treatment with water. The phosphorylating enzymes, which are not water-soluble, remain bound to the particles, as do the enzymes of photolysis.

*In vivo*, photolysis is linked (figure 3) either with phosphorylation, resulting in the production of ATP and the reconstitution of water, or with $CO_2$ fixation, resulting in the evolution of oxygen and the reduction of $CO_2$ to the level of carbohydrate (equations *6*, *7* and *3*). $CO_2$ fixation requires the participation of all three groups of enzymes, phosphorylation requires two, whereas photolysis of water can proceed without the others provided an artificial hydrogen acceptor is supplied. The last process, the well-known Hill reaction (equation *1*), provides a convenient method for measuring the activity of the enzymes concerned in the photolysis of water, under non-physiological conditions when neither photosynthetic phosphorylation nor $CO_2$ fixation takes place.

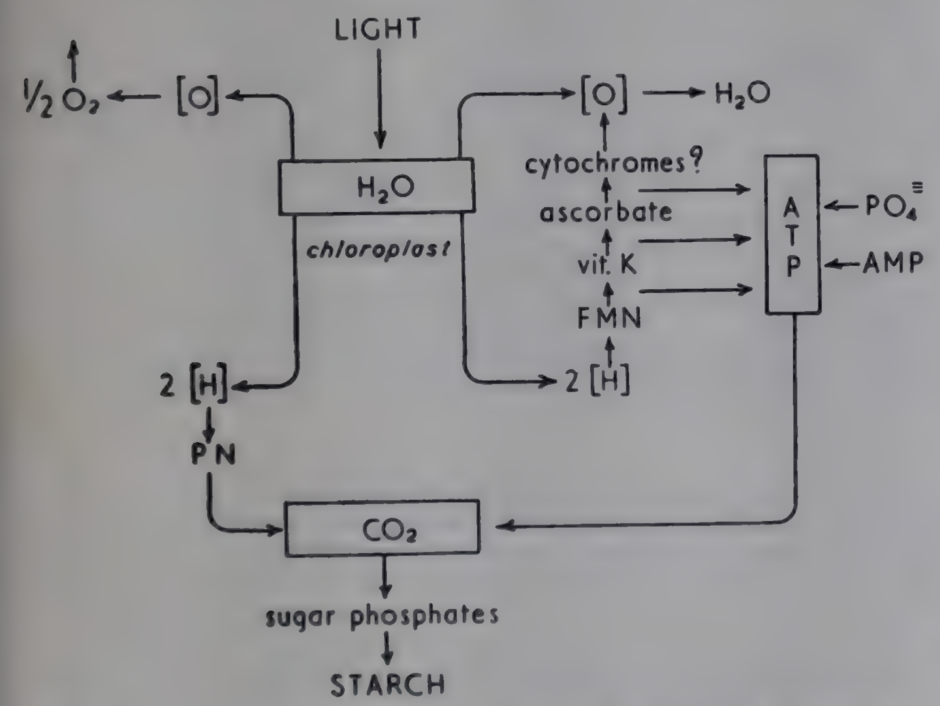


FIG. 3. — Scheme for photosynthesis by isolated chloroplasts. Photolysis of water (center) leading either to ATP synthesis and the reconstitution of water (right) or to $CO_2$ reduction (below) linked to oxygen evolution (left).

This postulated increasing order of complexity is supported by the experimental separation of the three chloroplast reactions (equations *1*, *2*, and *3*) by means of differential inhibitors. Chloroplast preparations capable of carrying out complete photosynthesis were used in three parallel series of experiments in which $CO_2$ fixation, photosynthetic phosphorylation and photolysis (Hill reaction) were measured separately. It was possible to inhibit a more complex phase of photosynthesis without affecting the simpler one which preceded it and, conversely, inhibition of a simpler phase of photosynthesis was invariably paralleled by an inhibition of the more complex phase which followed it. Thus iodoacetamide (4) and arsenite ($2 \times 10^{-3}$ M) inhibited $CO_2$ fixation but not photosynthetic phosphorylation or the photolysis reaction. Methylene blue ($10^{-5}$ M) inhibited both $CO_2$ fixation and photosynthetic phosphorylation but not the photolysis reaction. On the other hand, *o*-phenanthroline ($10^{-4}$ M) which inhibited photolysis also inhibited photosynthetic phosphorylation and $CO_2$ fixation.

As shown in figure 3, it is envisaged that the recombination of the products of decomposition of water in photosynthetic phosphorylation (equation *5*) proceeds in several successive steps, which together constitute an ,electron ladder' analogous to that discussed for respiration by Lipmann (18). Of the catalysts of photosynthetic phosphorylation, $Mg^{++}$ probably has a function in the transfer of phosphate, whereas FMN, vitamin K, and ascorbate could serve as electron carriers in the 'electron ladder' shown in figure 3 (see discussion in 1). The identity of the electron carriers above ascorbate is unknown, but they may very likely prove to be components of a cytochrome system.

The determination of the quantum efficiency of the conversion of light into pyrophosphate bond energy is under consideration. It is evident that phosphorylation, to be efficient, must convert the energy of one quantum of red light (43 kcal.) into the energy of more than one pyrophosphate bond (12 kcal.).

*General conclusions*

The elucidation of the detailed mechanism of photosynthesis embracing the transformation of light into chemical energy, and $CO_2$ and water into carbohydrates and oxygen, must, in the final analysis, rest on concordant data derived from experimental approaches at different levels : cellular, subcellular and molecular. The attainment of complete photosynthesis in a cell-free system offers special opportunities for investigating the process divorced from respiration (and other metabolic activities requiring molecular oxygen) and limits the identification

of the enzymes concerned to those contained in the chloroplasts. These subcellular structures have already been shown to be susceptible of further fractionation and quantitative biochemical study.

In the short time since complete extracellular photosynthesis by chloroplasts was established, no sufficient experimental evidence has as yet been accumulated to permit a detailed comparison with conclusions derived from other approaches to photosynthesis (19). A preliminary comparison reveals so far certain differences and similarities. The lack of inhibition of the photolysis reaction of isolated chloroplasts by sulfhydryl group inhibitors, especially arsenite, renders it unlikely that thioctic acid is, as postulated by Calvin (19), the electron acceptor in the primary photochemical act. Our data suggest that one or more sulfhydryl compounds participate in the later phases of photosynthesis. As for similarities, some of the same phosphorylated compounds were identified as products of photosynthesis by isolated chloroplasts and by whole cells. It would be premature, however, to conclude on the basis of the limited evidence available thus far, that the carbon cycle proposed by Calvin (19) accounts for the transformation of $CO_2$ into sugars by isolated chloroplasts. One conclusion seems inescapable. Granted the validity of our basic concept that the chloroplast of green plants is the special cytoplasmic structure in which are localized all the photosynthetic reactions from $CO_2$ and water to carbohydrates and oxygen (1, 4), then it follows that experiments with isolated chloroplasts, now in progress, should prove an important check on the validity of conclusions drawn from work with whole cells, single enzymes and model systems (19, 20).

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# Enzymatic mechanisms and biological significance of transmethylation reactions (*)

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(*Received* 7 *May* 1955)

In the last twenty years it has become increasingly clear that the living cell utilizes certain basic reaction patterns for the fulfillment of its metabolic needs. One such basic biochemical mechanism is 'group' transfer and indeed it would seem that our understanding of intermediary metabolism can be clearly related to the elucidation of an ever increasing variety of group transfer reactions and their interrelationships.

The early observations of His (1), of Hofmeister (2), of Maasen (3), and of Riesser (4) formed the background which led Challenger and Higginbottom (5), almost twenty years ago, to formulate with some precision the concept of biological transmethylation. Unequivocal evidence for the existence of transmethylation reactions was obtained through the brilliant work of du Vigneaud *et al.* (6). These investigators by means of ingenious utilization of isotopic tracers technique have proved beyond all reasonable doubt that the $—CH_3$ group is transferred as a unit from a compound acting as a methyl donor to a methyl acceptor compound (7). Although the work of the Cornell School has been largely limited to mammalian and avian species, evidence has been recently obtained by Byerrum (8) and Challenger (9), that transmethylation reactions play a major role in the biosynthesis of methylated compounds in plants and fungi. In bacteria, on the other hand, it appears as if transmethylation reactions are of minor importance. The discovery of transmethylation reactions in the mammal stems directly from the finding that in the absence of certain vitamin factors, later identified as Vitamin $B_{12}$ and folic acid, the diet must be fortified with a supply of compounds containing 'biologically labile' methyl groups; it was deduced, therefrom, that the adult mammal is not capable of synthesizing the methyl group. In the last few years this conclusion has been modified as a result of experiments which have disclosed that the mammal, when adequately supplied with vitamin $B_{12}$ and FA or its derivatives, is able to carry out *de novo* synthesis of the compounds containing biologically labile methyl groups (10, 11, 12, 13). A detailed discussion of this facet of methyl group metabolism would lead far afield for it is impossible to discuss *de novo* synthesis of methyl groups without relating it to the metabolism of 'one carbon' fragments and to the biological role of folic acid and vitamin $B_{12}$ (14). Some aspect of this problem will no doubt be discussed by Dr. Verly in this symposium. I wish to comment only on two points at this time.

Although a large amount of work has been directed towards the elucidation of the metabolic pathways, intermediates and coenzymes involved in the *de novo* synthesis of the methyl group from 'one carbon' fragments, the problem is still largely unsolved. One of the steps, presumably the last one, in the biosynthesis of a methyl group must be the reduction of a hydroxymethyl group. Such a reaction is suggested by the ready interconversion of the β-carbon of serine to the methyl group of choline and of thymine (15). In order to write a reaction mechanism for this reduction and regardless of the intermediates involved in this conversion (cystathionine, hydroxymethylhomocysteine, hydroxymethyltetrahydro-folic acid) it becomes necessary to postulate a series of hypothetical steps, some of which are without biochemical precedent. The first step might consist of the elimination of the —OH group (or of a phosphate group) through condensation of the hydroxymethyl compound (or a phosphorylated derivative thereof) with a suitable acceptor :

$$\text{R-CH}_2\text{OH} + \text{H-R}_1 \rightarrow \text{R-CH}_2\text{-R}_1 + \text{H}_2\text{O} \qquad (1)$$

where R and $R_1$ might be N, S or C atoms.

The next step would require a reductive cleavage (see also 16) as follows :

$$\text{R-CH}_2\text{-R}_1 \xrightarrow{+\,2\,\text{H}} \text{R-CH}_3 + \text{H-R}_1 \qquad (2a)$$

or :

$$\text{R-CH}_2\text{-R}_1 \xrightarrow{+\,2\,\text{H}} \text{R-H} + \text{R}_1\text{-CH}_3 \qquad (2b)$$

(*) The several investigations carried out by the author at Western Reserve University were aided by grants from the American Cancer Society and the William Waterman Fund for the Combat of Dietary Disease, and it is a pleasure to acknowledge their generous support.

The following abbreviations will be used in this paper : FA : folic acid *i. e.* pteroylglutamic acid ; AME : S-adenosylmethionine *i. e.* active methionine ; MAE : methionine activating enzyme ; TMA : thiomethyladenosine ; IP : orthophosphate ; ATP : adenosine triphosphate ; DMPT : dimethylpropiothetin ; DPN : diphosphopyridine nucleotide ; ARPPR : adenosine-ribose-pyrophosphateribose.

Numerous lines of investigation suggest the participation of FA or one of its reduced derivatives in this reaction sequence.

The concept that a single coenzyme might catalyze a biochemical transformation requiring more than one step is not entirely new. Some years ago on the basis of a chemical analogy Lipmann suggested that thiamine might function in precisely a similar manner in the oxidation of pyruvate (17). Although further developments did not support this particular interpretation of the mode of action of thiamine the concept that a single coenzyme might participate in a multistep reaction derives support from recent developments in the understanding of the biochemistry of uridine coenzymes and their role in the biological transformation of hexoses, such as the synthesis of galactose and glucuronate from glucose (18, 19).

Undoubtedly once the methyl group is formed *de novo*, transmethylation reactions occur forming other methyl compounds, but it is not certain whether 'one carbon' utilization leads to the biosynthesis of only one or of a variety of 'labile methyl compound'. In the guinea pig it has been shown that the synthesis of the methyl group of methionine proceeds by a pathway not requiring the formation of choline or betaine methyl groups (20). Other evidence is available which suggest direct synthesis of the methionine methyl group from 'one carbon' precursors; moreover, there are no data indicating the direct *de novo* synthesis of the methyl groups in compounds other than methionine. While it seems unlikely that there are many parallel pathways for the synthesis of the 'biologically labile' methyl compounds, further work will be required before it is definitely established that there is only one biological pathway for such a *de novo* synthesis.

By use of the nutritional approach, it was recognized that naturally occurring methylated compounds can be divided into two large groups : (*a*) the compounds containing a methyl group which is 'biologically labile', that is compounds which can act as methyl donors; and (*b*) compounds in which the methyl group is 'biologically stable' or inert. The first group is by far the smaller of the two and only four naturally occurring compounds may be classed as biological methyl donors; these are methionine, dimethylpropiothetin, betaine, and choline (*).

It has also been established that betaine and dimethylthetin function as methyl donors, both *in vivo* and *in vitro* only in the presence of homocysteine and all the evidence indicates that these compounds are involved in the synthesis of methionine through transmethylation. Choline itself should be considered only a potential methyl donor since it has been shown that a methyl group becomes 'mobile' (*) only after oxidation of choline to the corresponding aldehyde or to betaine (24). Furthermore, it is also established that the methyl-group found in choline, creatine, anserine, methylnicotinamide, epinephrine and other methylated derivatives are derived directly from the methyl group of methionine (6). For plants the same situations seem to hold as it has been shown that the methoxy group in lignins and the N-methyl groups in caffeine, hordenine, nicotine and other alkaloids are derived from methionine (8, 25, 26, 27).

An examination of the characteristics of the compounds which can function as source of labile methyl group reveals important differences, both in their chemical and physiological properties. In methionine, the sulphur atom has a pair of unshared electrons; on the other hand, dimethylpropiothetin, methioninemethylsulfonium, and betaine are 'onium' compounds in which this electron pair is shared with a carbon atom in a covalent bond. This clearcut chemical difference is reflected in a parallel difference in the biochemical behaviour of these compounds as methyl donors in enzymatic transmethylation reactions. Whereas the « onium » compounds are able to act as methyl donors for suitable methyl acceptors in the absence of energy sources, transmethylation systems which utilize methionine as the methyl donor require the participation of ATP (28, 29).

The role of ATP in such reactions is related to the activation of methionine (30) as described in equation *3*.

$$\text{L-methionine} + \text{ATP} \xrightarrow[\text{Mg}^{++}]{\text{GSH}} \text{S-adenosylmethionine} + 3\ \text{orthophosphates} \quad (3)$$

S-adenosylmethionine is a sulfonium compound characterized by the fact that, unlike methionine itself, it can function directly as a methyl donor. Thus it becomes clear that in a mechanistic sense the difference between the methyl 'onium' compounds and methionine as methyl donors is only apparent; methionine, in fact, undergoes conversion to a sulfonium compound prior to the release of its methyl group.

The correctness of the proposed structure of S-adenosylmethionine has been confirmed recently by total synthesis (31). When tested enzymatically, the synthetic material was between 40 and 50 % as active as the natural material (32). The synthetic material was DL with respect to the α-amino group and, presumably, it was also racemic at the sulfonium center. Therefore, the finding that the synthetic material is approximately

(*) Recent work (21) indicates that methionine methylsulfonium, a thetin like analogue of methionine found in cabbage (22) and in a variety of plants (23) may also function as methyl donor *in vivo*. Furthermore compounds chemically related to betaine such as carnitine, butyrobetaine etc. may function similarly, perhaps through the intermediation of betaine itself.

(*) The increasing complexity of our knowledge of transmethylation reactions and the growing understanding of their mechanism requires some clarification of the terminology. The term 'labile methyl compound' as defined by du Vigneaud relates to the ability of the compound to furnish in the diet 'methyl groups' for various synthetic reactions (33). This terminology is widely used and it is very desirable that is should be maintained, albeit restricted to the nutritional concept, as originally proposed. Mechanistically it might be convenient to define as 'mobile' the methyl group attached directly to a neutral 'onium' pole for reasons that will be come apparent.

50 % as active as the natural product is open to two interpretations which may be resolved by investigations, currently under way, of the biological activity of the optical isomers.

Having established that in all cases where the methyl group is 'mobile' it is attached directly to an 'onium' pole, it becomes of great interest to consider in more detail the possible reasons for this mobility. It has been emphasized (34) that the change in valence which accompanies the transfer of a methyl group from a methyl 'onium' compound to a methyl acceptor is in reality a reduction and that the energy of the « onium » bond is related to this change in the oxidation state. However, this analysis of the transmethylation reaction and related cleavages of 'onium' compounds is incomplete and rather unsatisfactory from a thermodynamic view-point. In reality, reduction of a sulfonium or quaternary ammonium atom must be accompanied by a concomitant oxidation. In aqueous solutions, such as prevail in biological systems, the concomitant and similar oxidation is the conversion of $H_2O$ to $H_3O^+$. Therefore for the purpose of arriving at an understanding of the energetics of the reaction a more complete and possibly a more useful analysis follows (35).

The transfer of an alkyl group from a sulfonium compound to form a thioether is typified by the formation of methionine from dimethylthetin and homocysteine as described by reaction *4* :

$$\left.\begin{matrix}CH_3\\CH_3\end{matrix}\right> S^+\text{-}R + H\text{-}S\text{-}R_1 + H_2O \rightleftarrows CH_3\text{-}S\text{-}R + CH_3\text{-}S\text{-}R_1 + H_3O^+ \qquad (4)$$

where $R = CH_2\text{-}COO^-$; and $R_1 = CH_2\text{-}CH_2\text{-}CHNH_2\text{-}COOH$. The overall reaction can conveniently be broken down in two partial steps : *4a* and *4b*.

$$\left.\begin{matrix}CH_3\\CH_3\end{matrix}\right> S^+\text{-}R + H\text{-}S\text{-}R_1 \rightleftarrows \left.\begin{matrix}CH_3\\H\end{matrix}\right> S^+\text{-}R + CH_3\text{-}S\text{-}R_1;\quad \Delta F_0(^*) = 0 \qquad (4a)$$

$$\left.\begin{matrix}CH_3\\H\end{matrix}\right> S^+\text{-}R + H_2O \rightleftarrows CH_3\text{-}S\text{-}R + H_3O^+;\quad \Delta F_0 = 0 \qquad (4b)$$

$$\left.\begin{matrix}CH_3\\CH_3\end{matrix}\right> S^+\text{-}R + H\text{-}S\text{-}R_1 + H_2O \rightleftarrows CH_3\text{-}S\text{-}R + CH_3\text{-}S\text{-}R_1 + H_3O^+;\quad \Delta F_0 = 0 \qquad (4s)$$

As is well known when $H^+$ (**) is formed in a reaction the $\Delta F$ will differ from $\Delta F_0$ by —1358 calories for unit increase in the pH; thus assuming that $\Delta F_0 = 0$, at physiological pHs the $\Delta F$ for reaction *4* is —10 000 calories or thereabouts.

(*) Standard $\Delta F_0$ is defined with all reactants at 1 M concentration and liquid water at 25° C.

(**) The terminology $H^+$ is conventionally used to represent the molecular species known to exist in aqueous solution as $H_3O^+$.

It is noteworthy that in both partial reactions *4a* and *4b*, one may assume a $K_{eq}$ close to unity. In reaction *4a* this is because there is little change in the electronic configuration of the reactants. In reaction *4b* a $\Delta F_0 = 0$ is equivalent to stating that the $pK_a$ of compound of the structure $\left.\begin{matrix}R_1\\R_2\end{matrix}\right> S^+\text{-}H$ is zero. The fact that compounds of this general formula are not known to exist in aqueous solution indeed indicates that the $pK_a$ is zero or lower. Should the $pK_a$ be lower, then the $\Delta F_0$ would become negative and this would correspondingly lower the $\Delta F$ of reaction *4b* (and hence of reaction *4*).

Recent work in the author's laboratory has centered around transmethylation reactions from methyl 'onium' compound and the enzymatic mechanism of formation of S-adenosylmethionine and its biochemistry. The transfer of the methyl group of AME has been studied using both nicotinamide and guanidinoacetate as methyl acceptors (36, 37). Two separate enzymes have been obtained which are capable of catalyzing the formation of N-methylnicotinamide and of creatine respectively. The properties, as well as some of the kinetic characteristics of these enzyme systems will be discussed.

It has been assumed for many years that homocysteine is one of the products in transmethylation reactions in which methionine is the methyl donor. The finding that in these reactions AME, rather than methionine itself, is the actual methyl donor logically leads to the supposition that adenosylhomocysteine rather than homocysteine should be one of the products of the reaction. This deduction has been verified and confirmed experimentally in our laboratory and adenosylhomocysteine has been prepared enzymatically and isolated (38). Its structure has been confirmed by synthesis (39). Further investigations as to the metabolism of this new amino acid may be of interest; the structure of adenosylhomocysteine invites speculation as to its participation in the inter-conversion of S-containing amino acids and perhaps, in the synthesis of the methyl group from one-carbon precursors (32).

The finding that AME contains adenine, pentose and methionine immediately suggested a relationship to thiomethyladenosine a nucleoside isolated from yeast many years ago by Suzuki *et al.* (40) and synthesized recently by Baddiley (41) and Satoh Makino (42). The origin of the thiomethyl group in that nucleoside was entirely obscure until Schlenk *et al.* (43) recently were able to demonstrate that the methyl group in TMA is derived from methionine by a process which these authors term transthiomethylation (see also 44). As is well known, sulphonium compounds decompose under a variety of conditions giving sulphides; therefore, it appeared reasonable to propose that thiomethyladenosine may be derived from AME by cleavage of the bond between the sulphur and the γ-carbon atoms. In collaboration with Baddiley and Jamieson (45), it has been shown that upon cautious hydrolysis AME breaks down chemically to thiomethyladenosine and homoserine. It has not yet been conclusively shown whether this type of chemical cleavage has a biological counterpart, but the work of Schlenk and of Schmidt (46) would strongly suggest that this is the case.

It might be of interest to discuss another type of enzymatic cleavage of sulfonium compounds, the cleavage of dimethylpropiothetin by a particulate enzyme obtained from *P. fastigiata* (47). The products of the reaction are dimethylsulfide, acrylic acid and $H^+$. This reaction is of interest from several points of view. Energetically it may be considered that in a cleavage of this type, the energy of the sulfonium bond is preserved in part, at least, in the double bond of acrylic acid. The natural occurrence of the substrate, DMPT, in marine algae was first discovered by Prof. Challenger and his collaborators, who also surmised that the liberation of dimethylsulfide which is observed in algae might be enzymatic (48). The amount of DMPT in *P. fastigiata* is very high indeed (49) and the enzyme, even in the relative crude form in which it has been studied, is exceedingly active. This relationship suggests that some important biological function must be performed by DMPT and the cleavage enzyme, but unfortunately we have as yet not been able to obtain evidence which would indicate what this function might be.

Before turning to the synthesis of AME, I would like to explore briefly other types of cleavage of 'onium' bonds, such as those catalyzed by thiaminase and DPNase, even though these reactions, strictly speaking, fall outside the main topic of this discussion.

As has been pointed out before (50, 35), the transmethylation reaction represents the simplest example of alkyl transfer from an 'onium' pole. The similarity between transmethylation reaction and other cleavages of alkyl 'onium' bonds and the broad physiological significance of these reactions has been stressed by Woolley who has made significant contributions both at the theoretical and at the experimental level (34, 51, 54).

Up-to-date transmethylation reactions represent the situation in which it has been most clearly recognized that the cell utilizes the energy of the 'onium' bond for biosynthetic reactions; however, by means of model systems, it may be shown that the 'onium' bonds in thiazolium and pyridinium compounds are potentially available for similar purposes. Thus the enzyme thiaminase, under appropriate conditions, catalyzes transfer of the methyl pyridimidine moiety of thiamine to suitable acceptors which may be a variety of secondary or tertiary amines (52). Similarly the enzyme DPNase has been shown to catalyze the transfer of the ARPPR moiety of DPN to other amines (53, 59). It is not possible to decide from experiments of this type what is the true biological role of DPNase and thiaminase respectively, but the concept that these enzymes are involved in biosynthetic mechanisms is very attractive.

The formation of AME from methionine and ATP possess a number of unusual features. As is well known, the key position of ATP in biological synthesis has hitherto been attributed to the release of considerable amounts of energy in the cleavage of its two pyrophosphate bonds. Thus the role of ATP is generally related to its ability to function as a phosphate donor. On the other hand, the bond between the ribose and the polyphosphate chain in ATP is usually considered to be relatively low in energy content. Thus a point of interest in the mechanism of formation of AME lies in the fact that a new energy rich bond is formed apparently at the expense of the energy-poor ester linkage between the ribose and the phosphate residue in ATP.

A further point of interest lies in the fact that in the course of the activation reaction catalyzed by a partially purified enzyme system obtained from rabbit liver (30), methionine activitating enzyme (MAE), all three phosphates of ATP appear as orthophosphate. This can only be explained by assuming secondary hydrolytic reactions and clearly indicates that the reaction mechanism is complex. Recent studies (55) have provided further information about the intermediary steps (*).

It was found that the rabbit liver preparation prepared as previously described, contains 8 units (**) of pyrophosphatase for each unit of methionine activating enzyme. In the course of purification the ratio of pyrophosphatase to MAE was lowered to about 0.2; the synthesis of AME catalyzed by this preparation was markedly activated by the addition of highly purified yeast pyrophosphatase (***). In view of the great specificity of pyrophosphatase, this observation indicates that pyrophosphate is formed in the course of the overall reaction, and furthermore, that in the absence of added pyrophosphatase, it accumulates in quantities sufficient to inhibit the synthesis of AME. This interpretation was supported by the finding that added pyrophosphate is inhibitory at low concentrations (0.001 M or less). In fact, using the purified preparation of MAE, pyrophosphate was identified as one of the reaction products, appearing in amounts equimolar with AME and orthophosphate. This stoichiometry has been observed only with short incubation periods. With increasing extent of reaction, the accumulating pyrophosphate inhibits the enzyme system while it itself undergoes slow hydrolysis, and a steady-state is soon attained at a pyrophosphate concentration of about 1 $\mu$-mole/ml.

In order to further elucidate the reaction mechanism, it was thought desirable to determine which two of the three phosphates of ATP give rise to pyrophosphate. For this purpose, ATP labelled equally in the two terminal positions with $^{32}P$ was prepared, and used as a substrate for the purified MAE. Pyrophosphate formed during the reaction was isolated by standard chromatographic procedures; the ratio of the specific activity of the orthophosphate derived from the pyrophosphate by acid hydrolysis to the specific activity of the 2 labile phosphates in ATP was determined. This ratio was found to lie close to 0.5. This establishes conclusively that one of the two phosphates in pyrophosphate is derived from the proximal phosphate in ATP; the reaction mechanism shown in equation *5* is consistent with this and other observations and may be adopted as a working hypothesis.

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(*) The following section has been prepared in collaboration with Dr. J. Durell, to whom the author wishes to express his real appreciation for many helpful discussions relating to the energetics and thermodynamics of transmethylation reactions.

(**) 1 unit of pyrophosphatase = 2$\mu$-moles of phosphate liberated in thirty minutes. 1 unit of MAE = 1$\mu$-moles of AME formed in thirty minutes.

(***) Generously supplied by Dr. L. Heppel.

$$\text{ARP-}^{32}\text{P-}^{32}\text{P} + \text{ME} \rightarrow \text{'ARP-}^{32}\text{P-ME'} + \text{I}^{32}\text{P} \qquad (5a)$$

$$\text{'ARP-}^{32}\text{P-ME'} \rightarrow \text{AME} + \text{P-}^{32}\text{P} \qquad (5b)$$

$$\text{P-}^{32}\text{P} \rightarrow \text{IP} + \text{I}^{32}\text{P} \qquad (5c)$$

$$\text{ARPPP} + \text{ME} \rightarrow \text{AME} + 3\ \text{IP} \qquad (5s)$$

The reversibility of these reactions, and the chemical nature of the postulated intermediate 'ARPP-ME' are under investigation.

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# Some aspects of mycological methylation and their relation to analogous processes in plants and animals, and to purely chemical reactions

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(*Received* 1 *August* 1955)

Dr. Cantoni has remarked that « in bacteria, it appears as if transmethylation reactions are of minor importance ». It would be of interest if he would elaborate this statement somewhat. I would agree that very little has been published on the subject, though some recent work suggests that the problem might well repay careful study. The literature of 50 years ago contains references to the production of volatile, odorous, unidentified products when cultures of various bacteria, including some which are pathogenic (and also a few yeasts) are treated with small quantities of arsenious oxide or sodium selenate. Cultures of the diphtheria bacillus on blood-agar-tellurite plates have recently been stated to evolve a garlic odour. I have not examined such plates personally and in some cases contamination by moulds may have occurred. In the absence of further information it must not be assumed that the odour was due to dimethyl telluride. Dr. Constance Higginbottom (1), Dr. Simons (2) and I examined several bacterial cultures containing arsenious oxide, sodium selenate or potassium tellurite in the hope of identifying and characterising volatile methyl derivatives of the metalloids, but without success. Very occasionally faint odours were observed but the picture was entirely different from that presented by various moulds. We paid particular attention to *Bacillus pyocyaneus* but obtained no methyl derivatives on aspiration of the gases from the cultures through suitable reagents. No doubt bacterial cells contain combined choline and possibly methionine but there is little information regarding the presence of other methyl derivatives. 5-methylcytosine occurs in the nucleic acids of the tubercle bacillus (3) and 5-hydroxymethylcytosine (which may be a precursor of the 5-methyl derivative) has been isolated from the products of the hydrolysis of the desoxyribonucleic acids of certain viruses which infect *Escherichia coli* (4). Evidence that cell suspensions of *Bacillus subtilis* can methylate uracil to thymine (identified by paper chromatography) has recently been presented (5). Addition of glycine increased the yield by 68 %. This is one of the few cases where, under controlled conditions, the methylating capacity of a bacterial cell has been demonstrated. Cultures of *Escherichia coli* can synthesise the riboside of 4-amino-5-carbonamido-iminazole and preparations of pigeon liver in presence of formate can supply carbon atom 2 of the purine ring, thus converting this compound to inosine-5-phosphoric acid (6).

A study of the synthesis of serine in cell suspensions of *Streptococcus faecalis* R (7) has emphasised the importance of formate, of pteroylglutamic acid (folic acid), its 10-N-formyl derivative and its 5-N-formyltetrahydro compound in this process. These compounds are, of course, closely connected with animal methylation processes. Animals and bacteria, have, it is clear, the same tools at their disposal and may well employ them for similar purposes. It is possible that N-5-hydroxymethyltetrahydrofolic acid (active formaldehyde) which has already been postulated as a co-factor for animal methylations may be concerned in the bacterial synthesis of serine. Further results will be awaited with interest. It is clear that a bridge is being built between the serine formate and purine metabolism of bacteria and animals and this may ultimately throw light on the methylation processes of bacteria.

Isotopic studies at Leeds (8-9) using methionine labelled with $^{14}C$ in the methyl group show that DL-methionine is an excellent donor of methyl groups in cultures of *Aspergillus niger* containing selenate, as shown by the high 'methylation percentage' (95-100) observed for the dimethyl selenide, counted as the mercuri chloride (that is, 90-100 % of the methyl groups of the dimethyl selenide were obtained from the labelled methionine). It seems probable that this amino-acid plays as dominant a part in mycological methylation as in the analogous animal processes.

Competition experiments in which unlabelled betaine, dimethylacetothetin chloride and dimethyl-β-propiothetin chloride were added to *A. niger* cultures containing selenate and labelled methionine, showed little alteration in the methylation percentage. As donors or as sources of the methyl group these compounds are in no way comparable with methionine and it seems that the thetin transmethylase which occurs in animal tissues is absent from the mould.

Dr. Cantoni refers to the possibility that the methylmethioninesulphonium ion may be a methyl donor. Experiments at Leeds (9) show that it is certainly a methyl source because, when labelled with $^{14}C$ in one methyl group and added to *A. niger* cultures containing selenate, the methylation percentage determined for the dimethyl selenide was 28-37. This methylation may have occurred either (*a*) directly or (*b*) after loss of one methyl group as $CO_2$ giving methionine. There is evidence that (*b*) occurs to some extent. On addition of unlabelled methylmethioninesulphonium iodide to *A. niger* cultures containing selenate and $^{14}CH_3$-DL-methionine, the methylation percentage was reduced from 95-100 (see above) to 59, due to competition with these cond methyl source, the methiodide. It would appear that this reduction is caused by unlabelled methionine arising from the loss of one methyl group from the methiodide which then competes with the

labelled methionine. There was conclusive evidence that unlabelled methionine was actually produced in this experiment.

It is therefore difficult to say whether the methiodide is a donor in its own right or only through breakdown to methionine.

Dr. Cantoni mentions that synthetic DL-S-adenosylmethionine (active methionine) is about half as active as the natural product in the enzyme systems which methylate guanidinoacetic acid and nicotinamide. It would appear to follow that D-methionine should not be a methyl donor in these systems.

It would be interesting to know whether an active methionine can be isolated from his enzyme systems to which the D-isomer has been added. Possibly the results of his experiments on « the biological activity of the optical isomers » may soon be available.

It has been shown in Leeds (9) that in *A. niger* cultures containing selenate the D, L- and DL-forms of methionine all give dimethyl selenide with a methylation percentage of 95-100. This is also the case when D-$^{14}CH_3$-methionine is in competition with the unlabelled L- isomer and *vice versa*. The D- and L-forms are therefore equally effective as methyl donors and it would appear that either (*a*) they are utilised directly with equal facility or (*b*) that the conversion of D-methionine ot the L-form proceeds at least as rapidly as the L- form can be utilised. Such a conversion may involve a D-aminoacid oxidase and reamination of the keto-acid. Other instances of the similar biological availability of the D- and L-methionines in biological systems have been reported (9).

Dr. Margaret Simpson and I (10) concluded that the enzymic elimination of dimethyl sulphide from dimethyl-β-propiothetin chloride is possibly not a normal feature of the life of the red marine alga *Polysiphonia fastigiata* as it does not occur till the weed is removed from the pools in which it grows, and exposed to air. It would be interesting to know if Dr. Cantoni has reached the same conclusion. It may be that the evolution of the sulphide is the first sign of damage to the alga and may be analogous to the evolution of trimethylamine from choline in dead animal tissue. On the other hand Cromwell at the University of Hull (11) finds that the trimethylamine evolved by the healthy hawthorn blossom arises by enzymic decomposition of choline.

Dr. Cantoni is uncertain as to the biological function which is performed by the seaweed enzyme in the fission of the thetin. So, unfortunately are we. It seemed possible that the fission might bear some analogy with the thiaminase reaction discussed by him in his report and studied by Woolley. In that case the fission giving dimethyl sulphide could make available the cation $^{+}CH_2.CH_2.CO.OH$ which with ammonia or trimethylamine in the weed could give β-alanine or its betaine and in the first case $H^+$. The betaine might also arise by methylation of β-alanine by an algal transmethylase. Unfortunately, however, examination by colleagues of an old evaporated alcoholic extract of *P. fastigiata* by paper chromatography failed to reveal the presence of any β-alanine, either before or after acid hydrolysis of any peptides which might be present. The betaine was not sought for specifically. Work in this direction is, however, being continued with fresh extracts.

It was shown in Leeds several years ago that the mobility of the methyl group in betaine (12) and also in dimethylacetothetin (13) (anhydride $Me_2$-$S^+$-$CH_2$-$COO^-$) could be demonstrated by heating with methyl acceptors such as aromatic bases, when the monomethyl derivatives of aniline, *p*-toluidine, *p*-anisidine and other amines were formed. This methyl transfer has now been extended by Miss B. J. Hayward (14) to phenols and O-methylation achieved. When betaine or dimethylacetothetin (anhydride) are heated with phenol or β-naphthol, anisole $C_6H_5$-O-Me and β-naphthyl methyl ether are obtained. In one experiment phenoxyacetic acid $C_6H_5$-O-$CH_2$-COOH was also isolated. These purely chemical reactions present an interesting analogy with the phenomena of biological methylation.

*Note added in press* (14 November 1955). — Ericson and Carlson (*Arkiv Kemi*, 1954, **6**, 511) report the occurrence of β-alanine, free and combined, in some dozen marine algae; it is perhaps significant that the highest concentration was formed in *P. fastigiata*. This observation is of importance in relation to the suggestion made above and is receiving further consideration.

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# On the utilization of hydrogen isotopes for the study of the metabolism of the methyl group

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(*Received* 20 *September* 1955)

I wish to congratulate Dr. Cantoni on his excellent lecture on labile methyl metabolism. Dr. Cantoni has clearly distinguished between transmethylation and labile methyl synthesis. I remember that, when labile methyl synthesis was first demonstrated to occur from one carbon units such as formate or formaldehyde (1), it was suggested that transmethylation always occurred through such intermediates. Dr. du Vigneaud then insisted that transmethylation means a transfer of the carbon of the methyl group with its three hydrogens from one molecule, the methyl donor, to another one, the methyl acceptor. The concept of transmethylation precludes the existence, as intermediates, of substances like formate, formaldehyde or their equivalents which no longer possess three hydrogens attached to the carbon.

The problem was thus to prove that transmethylation reactions occur in living cells. The nutritional experiments which showed that, in the diet given to rats, choline could be replaced by methionine, or methionine by choline and homocystine, and the tracer experiments which followed either the carbon or the hydrogen of the methyl group from one molecule to another, were highly suggestive and consistent with the concept of transmethylation. It is very good to have a fair hypothesis that is not contradicted by any fact, but it is still better to have a proof of its correctness. The nutritional and tracer experiments that I have so far mentionned, were not sufficient to eliminate the alternative hypothesis of formate or formaldehyde being intermediates in, for instance, the synthesis of choline from methionine and aminoethanol.

Du Vigneaud and his coworkers have given the much needed proofs of the reality of one biological transmethylation (from methionine to choline). You all know the two classical experiments of du Vigneaud :

(*a*) In one experiment, rats were fed, over a long period, a diet containing, as the only source of labile methyl groups, methionine labelled with deuterium in the methyl group; the animals were then sacrificed and the choline isolated from the carcass. The deuterium concentration of the methyl of the carcass choline rose to about 90 % of the deuterium concentration of the methyl of the methionine fed (2). In this experiment, less than one third of the hydrogen atoms of the methyl groups were lost when the carbon migrated from methionine to choline; in other words, many carbon atoms migrated with their three hydrogens : this is, by definition, a transmethylation reaction.

(*b*) In another experiment, rats received methionine labelled with $^{14}C$ and deuterium in the methyl group; the ratio $D/^{14}C$ in the methyl group of the choline isolated from the carcass was identical with that of the methyl of the methionine fed; the conclusion is again that the carbon atoms of the methyl groups migrated from one compound to the other with their three hydrogens (3, 4).

When Dr. Cantoni says that methionine must be activated to S-adenosylmethionine to give its methyl group to an acceptor, it seems logical to admit that this sequence of bioreactions intergrates within the concept of transmethylation. But I wonder whether a direct proof of it might not be useful; I suggest to Dr. Cantoni to doubly label the methyl of the S-adenosylmethionine and to test, in his *in vitro* system, whether the isotope ratio in the methyl of the methylated acceptor is the same as in the labelled donor.

We know that the labile methyl group can be oxidized to $CO_2$ through the intermediary oxidation steps of formaldehyde and formate (5); we also know that these intermediates can be used for methyl synthesis (1). Between the labile methyl groups of two compounds two pathways seem possible : transmethylation, and oxidation followed by reduction. If one believes that, in a particular case, the reaction is one of transmethylation, it is still not possible to omit to prove that it is so.

I have recommended the use of the double labelling technique to prove transmethylation; I have now to add a few words of caution. To doubly label a methyl group, one has to use an isotope of hydrogen, either deuterium or tritium, and these isotopes do not behave like protium, hydrogen mass 1, which is nearly the only isotope present in ordinary hydrogen : the bond between C and H is much stronger when the hydrogen is deuterium than when it is protium, and still stronger when it is tritium.

If, as is the case in transmethylation reactions, there is no breaking of the C-H bonds, one may hope to have no isotope effect and, in this particular case, deuterium or tritium is a true tracer.

The situation is very different if some of the C-H bonds are split : the C-protium bonds are preferentially split and the C-D or C-T bonds are preferentially retained; finally the number of bonds broken is in excess of what the deuterium or tritium content of the reaction product indicates.

The possible magnitude of this isotope effect is really startling. In 1951, in Dr. du Vigneaud's laboratory, we injected subcutaneously to rats a mixture of $^{14}CH_3OH$, $CD_3OH$ and $CH_2TOH$ and we measured the $^{14}C$, D and T in the methyl groups of the choline isolated from the carcass; we knew that methanol was a precursor of the labile methyl group (6) and that conversion of methanol to choline methyl occurred through oxidation of the methanol methyl followed by reduction (4). If,

during these bioreactions, there had been no isotope effect, the ratio T/D ought to have been the same in the isolated choline methyl group as in the methanol methyl group; but we found it to be three times higher in the choline methyl than in the methanol methyl: the ratio $D/^{14}C$ in the choline methyls was 22 % and the ratio $T/^{14}C$ 72 % of what they were in the methanol methyl (7); this comparison gives an idea of the possible magnitude of the isotope effect in the very kind of biochemical reactions that we are studying in this colloquium.

This work on hydrogen isotope selection in connection with methyl metabolism which I initiated in 1951, was continued in Dr. du Vigneaud's laboratory by Dr. Rachele and later by Dr. Aebi from Switzerland. Dr. Rachele and E. Kuchinskas, with the collaboration of Dr. Eidinoff from the Sloan Kettering Institute, injected subcutaneously to rats a mixture of $^{14}CH_3OH$, $CH_2DOH$ and $CH_2TOH$; the difference between this experiment and the preceeding one is that $CD_3OH$ has been replaced by $CH_2DOH$; in the second experiment, the ratio $D/^{14}C$ in the choline methyls was 66 % and the ratio $T/^{14}C$ 84 % of what they were in the methanol methyl (8).

We shall try to interpret these experiments. When a hydrogen labelled methyl group undergoes oxidation, the isotope content of the reaction product depends on which hydrogen isotope has been used (deuterium or tritium) and on how the molecule has been tagged (whether one or the three hydrogens of the methyl group have been labelled). The results obtained with the $CD_3$ label are probably the most representative of what normally happens to the $CH_3$ group, because there is no competition, within the same methyl group, between the hydrogen atoms; but the oxidation rate of $-CD_3$ is smaller than the oxidation rate of the normal $-CH_3$. I recall Thorn's experiment on the oxidation rate of deuteriosuccinic acid by the succinic-dehydrogenase: the reaction rate was smaller for dideuteriosuccinic acid than for normal succinic acid, and still smaller for tetradeuteriosuccinic acid (9).

The reduction of the oxidation reaction rate due to the deuterium label, is important in experiments where the double labelling technique is used; the doubly labelled compound is usually prepared by mixing a carbon labelled species and a hydrogen labelled species, for instance a $^{14}CH_3$- species and a $CD_3$- species; when the methyl is oxidized, the reaction rates are different for the two molecular species and the ratio $D/^{14}C$ does not tell what happens to a normal $CH_3$- group. It would seem much better to have one molecular species labelled simultaneously in the carbon and the hydrogens, for instance a $^{13}CD_3$- compound; although the rate of oxidation of this species is certainly different from that of normal $^{12}CH_3$-, it might be hoped that the reaction mechanism would not be altered and that the change of $D/^{13}C$ ratio would be parallel to the change of $H/^{12}C$ ratio during the oxidation steps.

The least desirable label to use to trace the fate of a methyl group is $CH_2T$, because T is so different from protium; not only oxidation reaction rates are different for the $CH_2T$- and $CH_3$- species, but there is, within the grouping itself, a competition between protium and tritium. But that label, bad as it is, is the only one that can be used to solve certain problems; we had to employ it to study the biological origin of the methyl group of adrenaline.

In conclusion, I shall say that it is almost impossible to know by the isotope technique what happens, quantitatively and qualitatively, to the normal-$CH_3$ group on oxidation. Qualitatively, the best approach is to use the $^{13}CD_3$-label: it can probably tell the level of oxidation reached; but it does not tell how many methyl groups have undergone oxidation. Moreover, there is always the danger that oxidation of the methyl group followed by reduction, might be interpreted as transmethylation by people unaware of the isotope selection, because the heavy hydrogen isotopes are so firmly attached to the carbon.

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# Adaptation respiratoire : développement du système hémoprotéique induit par l'oxygène

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(*Reçu le* 11 *mai* 1955)

« Le chimiste pourra faire les produits de l'être vivant, mais il ne fera jamais ses outils, parce qu'ils sont le résultats de la morphologie organisée qui est hors du chimisme proprement dit, et, sous ce rapport, il n'est pas plus possible au chimiste de fabriquer le ferment le plus simple que de fabriquer l'être vivant tout entier. »

Claude BERNARD

Pour les oxydations cellulaires terminales, l'outil est le système hémoprotéique, et la citation garde encore toute sa valeur. A défaut de fabriquer l'outil, nous pouvons essayer de voir comment il est fait par la cellule.

La respiration de presque toutes les cellules qui vivent à l'air est catalysée par les enzymes du système Warburg-Keilin (1 à 3). On ne saura vraisemblablement jamais comment le monde vivant a développé le système qui lui permet d'utiliser l'$O_2$ comme accepteur d'électrons. On saura peut-être comment une cellule forme les hémoprotéines respiratoires. La formation, induite par l'$O_2$, de l'outil respiratoire chez la levure constitue le sujet de mon exposé.

La levure prolifère, dans un milieu adéquat, presque aussi bien en anaérobiose qu'en aérobiose (4, 25, 26). Cultivée en présence d'$O_2$, elle possède un système d'enzymes respiratoires qui n'est pas très différent de celui, par exemple, du muscle cardiaque. La même levure cultivée pendant un jour en absence d'$O_2$ ne possède pas d'enzymes respiratoires et, par conséquent, ne respire pas. L'$O_2$ induit la formation du système hémoprotéique qui peut se produire en absence de la division cellulaire. J'appellerai « adaptation respiratoire » l'ensemble du développement induit par l'$O_2$ dont l'effet majeur est l'accélération de la vitesse de la consommation de l'$O_2$. J'utiliserai le terme « biosynthèse induite » (5) en parlant des hémoprotéines individuelles. Je limiterai l'analyse de ce phénomène à la levure, matériel dans lequel il est le mieux connu (6 à 26). Il est probable que le même phénomène se produit chez les autres organismes qui peuvent vivre en présence et en absence d'air (27 à 30).

## DESCRIPTION DU PHÉNOMÈNE

Le fait que l'adaptation respiratoire peut avoir lieu en absence d'une source extracellulaire d'azote assimilable, donc de croissance, a considérablement facilité son étude. La levure cultivée au préalable en absence d'$O_2$ (levure anaérobie) est simplement mise en suspension dans un tampon contenant une source d'énergie et de carbone (par exemple du glucose), et aéré (figure 1). La

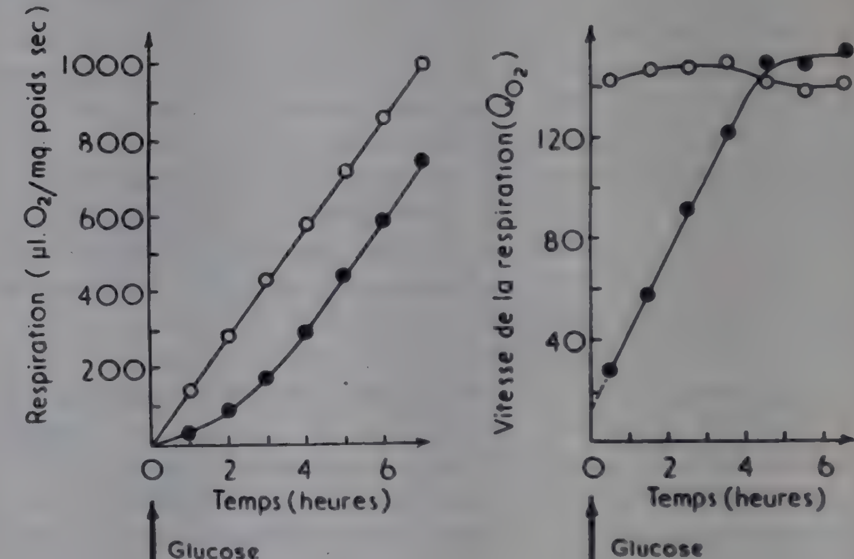


FIG. 1. — Adaptation respiratoire. La levure préalablement cultivée en aérobiose (cercles clairs) ou en anaérobiose (cercles pleins) est lavée, suspendue dans le tampon phosphate, puis aérée. Le glucose 0.007 M est ajouté au début de l'aération, c'est-à-dire 20 min. avant le début des mesures manométriques.

vitesse de la respiration augmente dans le temps tandis qu'elle reste constante chez une levure cultivée au préalable en aérobiose. Comme pour la respiration, qui est mesurée par sa vitesse ($dO_2/dt$, unités de $Q_{O_2}$ = µl.$O_2$/mg. poids sec/h.) l'adaptation respiratoire est mesurée par sa vitesse ($d^2O_2/dt^2$, unités de $A_{O_2}$ = µl.$O_2$/mg. poids sec/h$^2$). Le $A_{O_2}$ est donc une mesure de l'accélération du $Q_{O_2}$. Les $Q_{O_2}$ de la levure non adaptée et adaptée sont respectivement de 7 ± 3 et de 150 ± 10,

alors que les valeurs de $A_{O_2}$ peuvent varier selon les conditions expérimentales entre 0 et 30.

*Enzymes induits par l'oxygène*

La levure peut être broyée et les activités enzymatiques des extraits mesurées par les méthodes habituelles (tableau I). Ce tableau n'est pas destiné à comparer entre elles les valeurs des enzymes extraits d'une même levure, mais permet la comparaison des activités enzymatiques de deux levures. Le rapport des activités correspond, pour la plupart des enzymes, au rapport des concentrations en protéines enzymatiques des cellules intactes, adaptées et non adaptées. Les arguments en faveur de cette interprétation ont été discutés ailleurs (18, 25). Parmi les activités consignées dans le tableau, certaines représentent des systèmes composés d'au moins deux protéines distinctes (31 à 33) ce qui ne peut qu'augmenter le nombre d'enzymes sujets à l'adaptation respiratoire. Il est difficile de savoir, pour des raisons évidentes, si les très faibles activités de la levure non adaptée correspondent à des teneurs réelles et non à des oxydo-réductions non spécifiques. Un effort particulier a porté sur la cytochrome *c* oxydase. La teneur en cet enzyme est inférieure à 1/200 de sa concentration chez la levure adaptée.

Bien que l'oxygène induise le développement de tout un système multi-enzymatique, les biosynthèses individuelles ne sont pas synchrones (figure 2). La biosynthèse de la cytochrome *c* oxydase et probablement celle du cytochrome *c* évoluent simultanément avec l'adaptation respiratoire globale. Ces deux enzymes constituent donc les facteurs limitant l'accroissement du $Q_{O_2}$ de la cellule intacte, constatation en accord avec les mesures de leur nombre de rotations *in situ* (34, 3). Par contre, la succino-cytochrome *c* réductase ne semble pas participer activement à la respiration, car l'accroissement de son activité est nul dans l'intervalle où le $Q_{O_2}$ augmente de 5 à 50 (figure 2). Il est clair également que chez la levure le

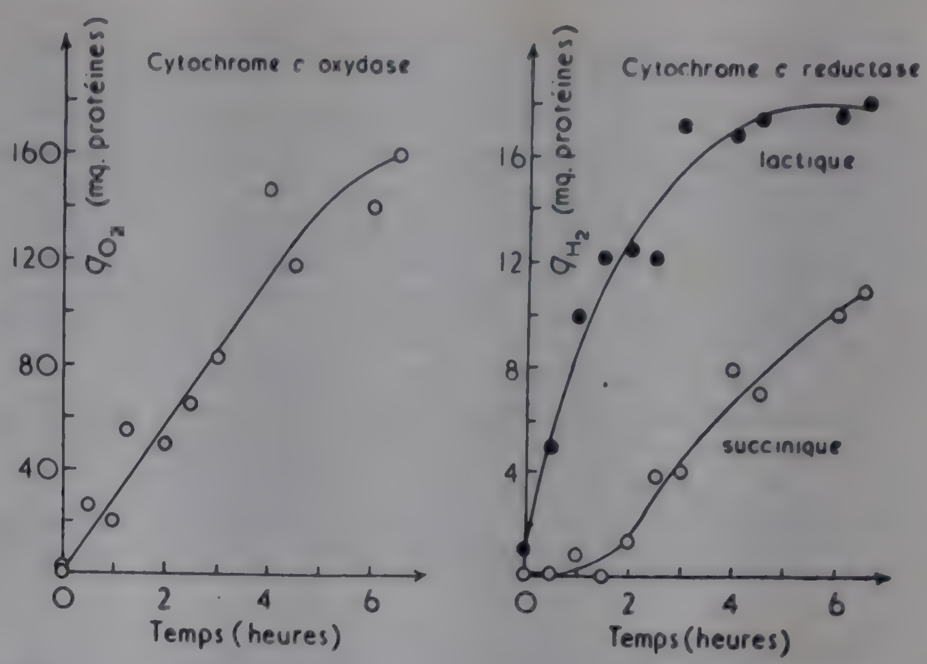


**Fig. 2.** — Accroissement, au cours de l'adaptation respiratoire, de la teneur en trois enzymes hémoprotéiques. Adaptation en glucose 0.007 M.

cycle tricarboxylique ne fait pas exclusivement partie du métabolisme aérobie (tableau I). Les résultats fournis par l'étude de l'adaptation respiratoire viennent ainsi appuyer les données obtenues par des méthodes entièrement indépendantes (3, 35).

Au développement des activités enzymatiques lors de l'adaptation correspondent des changements spectroscopiques que l'on peut observer directement sur la cellule vivante. On voit ainsi apparaître les cytochromes *a*, $a_3$, *b* et *c* absents chez la levure anaérobie. Des hémochromogènes natifs de la levure anaérobie, du type $b_1$ et $a_1$, disparaissent au fur et à mesure de la formation du système normal. Le rôle de ces hémochromogènes anaérobies n'est pas clair et leur contribution à la biosynthèse des cytochromes normaux non plus. Ils servent très probablement de précurseurs pour les groupes héminiques qu'ils cèdent, mais on ne sait pas s'ils

TABLEAU I

*Enzymes formés au cours de l'adaptation respiratoire*

| Enzyme | Activité de la levure | | Accroissement de l'activité |
|---|---|---|---|
| | Non adaptée | adaptée | |
| Cytochrome *c* oxydase (µl. $O_2$/h.) | < 1 | 180 | > 200 × |
| Cytochrome *c* peroxydase (Unités *cf.* 23) | < 0.03 | 1.6 | > 50 × |
| Catalase (Unités *cf.* 22) | < 0.3 | 10 | > 30 × |
| Cytochrome *c* (moles × $10^{-10}$) | env. 0.1 | 1.8 | env. 20 × |
| Lactico-cyt. *c* réductase (µl. $H_2$/h.) | env. 0.5 | 25 | env. 50 × |
| α-Glycérophosphate cyt. *c* réductase (µl. $H_2$/h.) | < 0.2 | 12 | > 50 × |
| DPNH-cyt. *c* réductase (µl. $H_2$/h.) | env. 1.6 | 64 | env. 40 × |
| Succino-cyt. *c* réductase (µl. $H_2$/h.) | env. 0.3 | 13 | env. 40 × |
| Aconitase (µl. *cis*-aconitate/h.) | 5 | 96 | 20 × |
| Fumarase (µl. fumarate/h.) | 120 | 800 | 7 × |
| Déshydrogénase malique (DPN) (µl. $H_2$/h.) | 20 | 110 | 6 × |
| Déshydrogénase *iso*citrique (DPN) (µl. $H_2$/h.) | 7 | 20 | 3 × |
| Alcool déshydrogénase (DPN) (µl. $H_2$/h.) | 83 | 35 | 0.4 × |

Les activités sont exprimées par mg. protéines de l'extrait total (16, 17, 18, 22, 23, 25).

contribuent ou non aux apoprotéines formées. De façon générale, l'adaptation respiratoire s'accompagne de relativement peu de changements dans la teneur globale en hémines, et sa vitesse n'est pas accélérée par l'addition de la ferroprotoporphyrine. Il faut donc la distinguer clairement des variations dans la constitution en enzymes respiratoires causées par la carence en hémines (36 à 39). Il ne faut pas conclure, cependant, que les hémines ne jouent aucun rôle dans le mécanisme de l'induction par l'$O_2$ ou dans la biosynthèse des apohémoprotéines.

La biosynthèse des hémoprotéines induite par l'$O_2$ concerne en premier lieu les parties protéiques des enzymes. Les analogues des acides aminés comme la *p*-fluorophénylalanine (40) inhibent l'adaptation respiratoire (figure 3) et la formation de la catalase (22). On

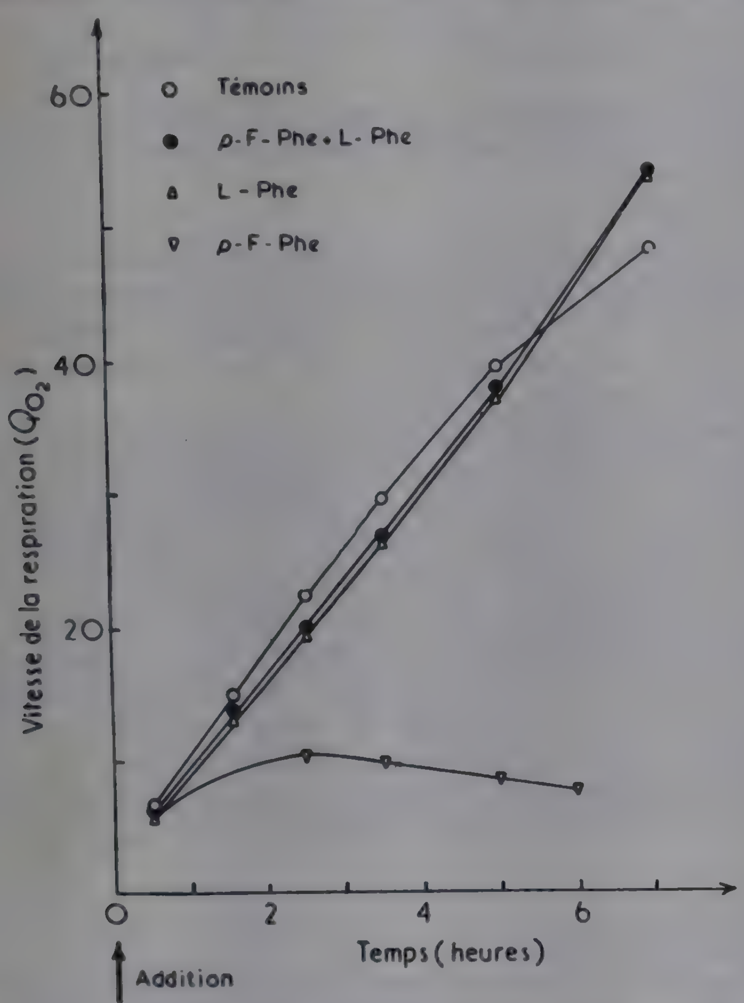


Fig. 3. — Inhibition de l'adaptation respiratoire par l'analogue d'un acide aminé (*p*-F-phénylalanine, 0.009 M. Suppression de l'inhibition par son homologue (L-phénylalanine, 0.009 M). Adaptation en glucose 0.17 M.

verra plus loin comment l'adaptation respiratoire est conditionnée par la présence d'un *pool* intracellulaire d'acides aminés. Etant donné que la biosynthèse des enzymes respiratoires n'est pas accompagnée de l'accroissement net de la quantité totale des protéines cellulaires, il faut en conclure soit que la somme de tous les enzymes formés est inférieure à 2 % des protéines, ce qui est extrêmement peu probable, soit que des dégradations compensatrices ont lieu simultanément.

### *Spécificité de l'induction par l'oxygène*

Parmi les nombreux accepteurs d'électrons et d'hydrogène qui peuvent oxyder une partie du système respiratoire, aucun ne remplace l'oxygène en tant qu'inducteur de l'adaptation respiratoire. On peut évidemment penser que la biosynthèse de la catalase et de la cytochrome *c* peroxydase n'est pas induite par l'oxygène moléculaire, mais par les peroxydes qui se forment en sa présence. On devrait pouvoir trancher cette importante question en utilisant comme inducteurs des peroxydes en anaérobiose. Les premières tentatives dans cette direction n'ont pas donné de résultats concluants (23).

Il est probable que le développement de tout le système hémoprotéique s'explique par une séquence d'inductions, l'oxygène induisant la biosynthèse de la cytochrome *c* oxydase, celle-ci induisant la biosynthèse du cytochrome *c* qui, à son tour, induit la formation des cytochromes *c* réductases, et ainsi de suite, conformément à l'idée de l'adaptation successive (41, 42). La chaîne des inductions suivrait donc la même voie que le transfert d'électrons et d'hydrogène dans la respiration, mais en sens inverse. Au moment où j'ai proposé cette interprétation, elle ne pouvait pas expliquer l'induction par l'$O_2$ de la biosynthèse du cytochrome *c* chez un mutant de la levure dépourvu de cytochrome *c* oxydase (18). Cette hypothèque a été levée récemment par la démonstration que l'$O_2$ induit chez ce mutant la cytochrome *c* peroxydase (23).

## INTÉGRATION DE L'ADAPTATION RESPIRATOIRE DANS LE MÉTABOLISME

Les enzymes respiratoires ont une fonction clé. On devrait donc s'attendre à ce que leur développement soit étroitement intégré dans le métabolisme de la cellule. L'adaptation respiratoire exige, comme toutes les biosynthèses, de l'énergie et des matériaux de construction. Nous allons d'abord étudier ces deux facteurs séparément, puis nous étudierons leurs interactions.

### *Relation entre le fonctionnement et la biosynthèse des hémoprotéines*

Ce problème peut d'abord être posé de la manière suivante : l'activité catalytique des enzymes respiratoires est-elle absolument indispensable à leur formation? Si la réponse est négative, on doit poser le problème autrement : l'activité catalytique des enzymes respiratoires influe-t-elle sur leur formation ? Par la première question nous voulons distinguer entre les deux hypothèses alternatives, celle qui implique que le développement du système hémoprotéique est causé par la respiration même et celle qui implique que ce développement est causé par l'$O_2$. Par la deuxième question, nous voulons savoir comment la respiration agit sur la biosynthèse des enzymes qui en sont responsables.

L'activité catalasique de la levure intacte et des extraits non-cellulaires est inhibée de moitié par le $NaN_3$, $1.1 \times 10^{-6}$ M. Par contre, pour inhiber la formation induite de la catalase, il faut 500 ou 1000 fois plus d'azoture. La catalase dont l'activité est réduite de plus de 99 % est synthétisée comme l'enzyme non inhibé (24).

L'inhibition par le KCN de l'adaptation respiratoire et de la biosynthèse de la cytochrome *c* oxydase fournit un résultat analogue. Le cyanure inhibe le fonctionnement des enzymes respiratoires de la levure (inhibition de 50 %

à $5 \times 10^{-6}$ M, *cf* 43). Il faut au moins 100 fois plus de l'inhibiteur pour réduire de moitié l'adaptation respiratoire. La cytochrome *c* oxydase dont l'activité est réduite de plus de 95 % est synthétisée normalement. Il est d'ailleurs vraisemblable que l'action inhibitrice du KCN sur l'adaptation respiratoire est indirecte et résulte de modifications du métabolisme général.

Les résultats obtenus sur l'inhibition de l'activité catalytique des hémoprotéines suggèrent que cette activité n'est pas une condition obligatoire de leur biosynthèse. Dans le cas des hydrolases inductibles, on est arrivé à la même conclusion en partant d'un type d'expérience différent et complémentaire (44 à 46). Cette conclusion est importante car elle rend peu vraisemblables les hypothèses sur la formation induite des enzymes, fondées simplement sur leur activité catalytique (47). Il faut cependant garder présentes à l'esprit les deux considérations suivantes : (*a*) La vitesse avec laquelle l'enzyme agit sur son substrat est au moins 1000 fois plus grande que la vitesse avec laquelle la cellule forme l'enzyme. Autrement dit, pendant que la cellule a formé une unité d'enzyme dont l'activité a été réduite de 99 %, cette unité d'enzyme a, néanmoins, effectué 10 fois son action catalytique. Il est donc très difficile, sinon impossible, d'apporter actuellement une preuve expérimentale de la formation de l'enzyme qui n'est pas actif. (*b*) Pour la sélection naturelle du système formateur de l'enzyme inductible, l'activité catalytique de cet enzyme a joué certainement un rôle capital. Si l'activité catalytique de l'enzyme n'est, peut-être, pas nécessaire à son ontogénèse, elle a été nécessaire à sa phylogénèse.

Ajoutons enfin que l'adaptation respiratoire peut être modifiée sans que le fonctionnement des enzymes respiratoires soit affecté. Ceci est évident car le premier processus est beaucoup plus complexe que le second.

On verra dans les paragraphes suivants comment le développement des hémoprotéines respiratoires est influencé par leur activité enzymatique.

### *Energétique de l'adaptation*

L'énergie nécessaire à la biosynthèse des hémoprotéines peut être fournie, en principe, soit par le métabolisme respiratoire, soit par le métabolisme fermentaire. Supposons que les matériaux de construction soient en excès et que la vitesse de l'adaptation respiratoire soit proportionnelle à l'apport d'énergie utilisable pour les biosynthèses :

$$A_{O_2} = \frac{1}{K'_1} \quad \frac{d\ \text{Energie}}{dt}$$

$$\frac{d\ \text{Energie}}{dt} = K_2\, Q_{O_2}^{\text{air}} + K_3\, Q_{CO_2}\ \text{ferm.}$$

$$A_{O_2} = \frac{K_2}{K'_1}\, Q_{O_2}^{\text{air}} + \frac{K_3}{K'_1}\, Q_{CO_2}\ \text{ferm.} \qquad (1)$$

Peut-on vérifier la validité de cette équation et déterminer la valeur des constantes $K'_1$, $K_2$ et $K_3$ ?

Lorsque la source d'énergie est constituée par une substance qui est métabolisée seulement par la voie respiratoire, on devrait trouver selon l'équation (*1*) que l'évolution de l'adaptation respiratoire dans le temps suit une cinétique exponentielle. Des expériences effectuées sur acétate ou sur éthanol montrent que c'est effectivement le cas. Remarquons que les cellules aérobies respirent sur éthanol ou sur acétate avec la même vitesse que sur glucose (figure 4 A). Cependant les cellules anaérobies s'adaptent lentement sur éthanol seul. L'addition d'une petite quantité de glucose, rapidement épuisée, donc insuffisante elle-même à assurer la respiration, permet l'adaptation et la respiration sur éthanol (figure 4 B). Cet effet d'amorçage s'explique par une contribution énergétique de l'utilisation du glucose. La vitesse de la respiration, accrue grâce à cette énergie, devient suffisante pour assurer l'adaptation dont la cinétique devient alors linéaire dans le temps. Les expériences effectuées avec des substrats non fermentescibles sont donc en bon accord avec l'équation (*1*) et confirment le point de vue

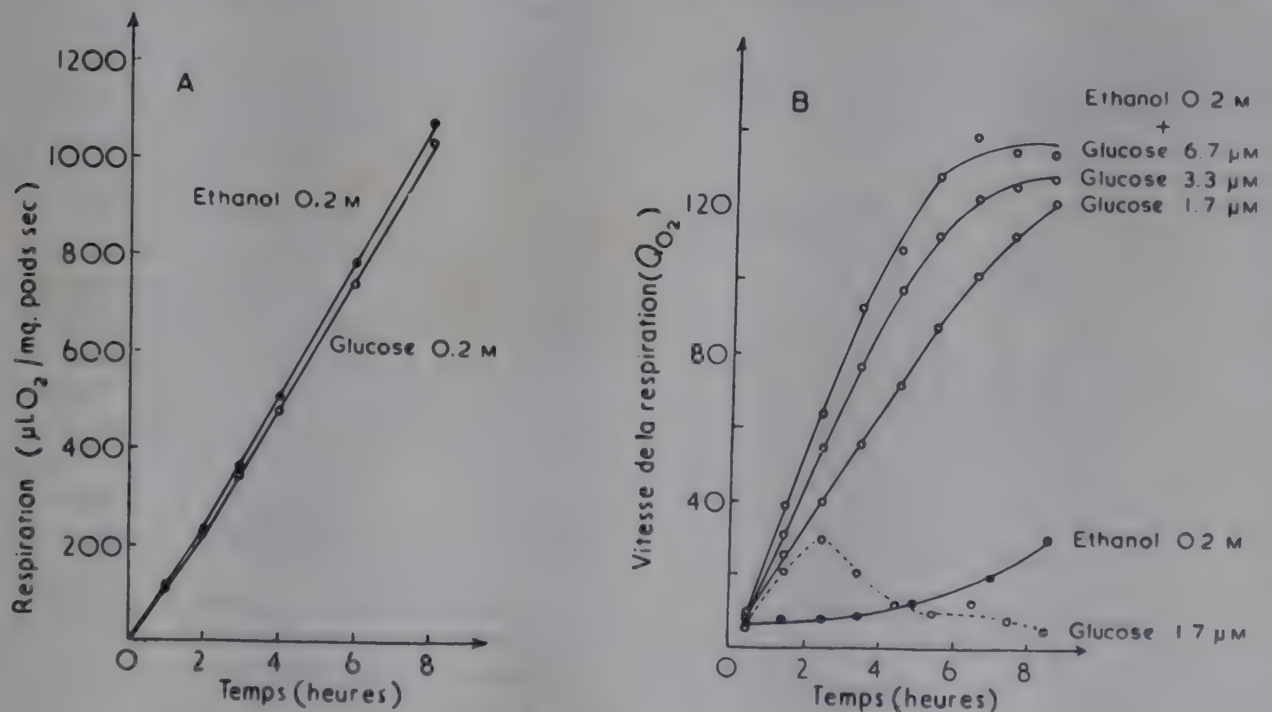


FIG. 4

A. — Respiration sur éthanol et sur glucose de la levure préalablement cultivée en aérobiose.
B. — Adaptation respiratoire sur éthanol seul et sur éthanol additionné d'une petite quantité de glucose.

sur une relation entre l'activité métabolique de l'enzyme et la cinétique de sa biosynthèse (48). Cet accord est seulement qualitatif et ne donne pas de valeurs numériques des constantes $K'_1$, $K_2$, $K_3$.

Un système à « double adaptation » permet une vérification quantitative. Etant donné que son principe est susceptible d'autres applications et que son exécution expérimentale est très simple, il me paraît utile d'en parler avec quelques détails. Prenons une levure cultivée en anaérobiose sur glucose et exposons-la à l'air et au maltose (ou au galactose) comme seule source d'énergie. Son $Q_{O_2}$ et son $Q^{air}_{CO_2}$ ferm. sont initialement petits ($<10$). Elle est doublement inadaptée car elle ne possède ni enzymes respiratoires ni enzymes nécessaires à la fermentation du sucre (« galactozymase » ou « maltozymase »). Ces enzymes sont inductibles et leur biosynthèse nécessite de l'énergie qui peut être fournie soit par la respiration soit par la fermentation (49). Tant que l'énergie est limitante l'évolution d'une double adaptation peut être comparée à la progression d'une cordée de deux alpinistes, la biosynthèse des hémoprotéines entraînant la biosynthèse des hydrolases, et vice-versa. Ajoutons, que les expériences de contrôle ont montré que les cellules anaérobies ou aérobies qui possèdent la maltozymase synthétisent les cytochromes et respirent sur maltose de la même façon que sur glucose. L'addition initiale du glucose qui est

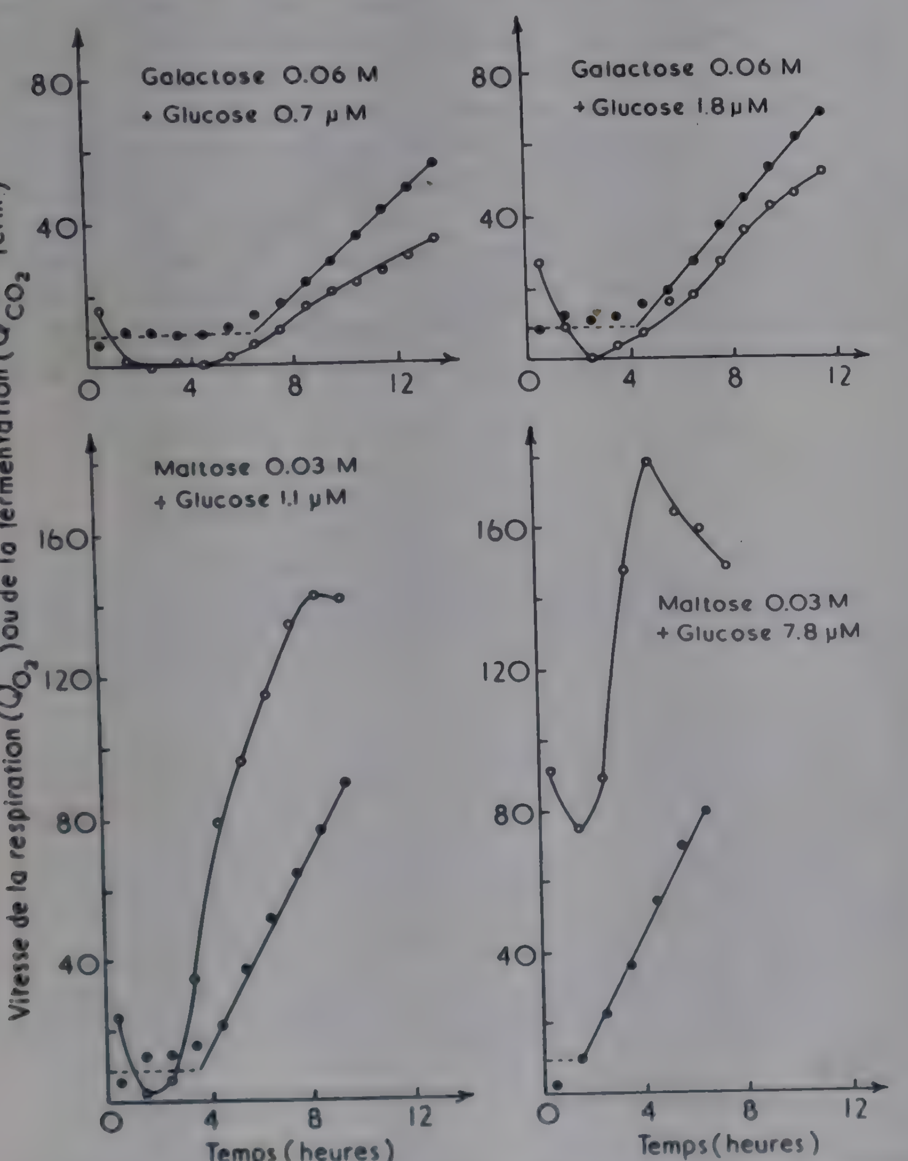


Fig. 5. — Doubles adaptations amorcées par le glucose. Vitesse de respiration : cercles pleins ; vitesse de fermentation : cercles clairs.

fermentescible d'emblée accélère l'adaptation (figure 5), comme elle le fait sur éthanol (figure 4 B). En faisant varier la quantité de glucose ajoutée, on fait varier au moment de « l'étape critique » (voir plus loin) les trois facteurs $A_{O_2}$, $Q_{O_2}$ et $Q^{air}_{CO_2}$ ferm. Ces trois facteurs sont liés par l'équation (*1*) qui peut être mise sous la forme suivante :

$$\frac{A_{O_2}}{Q_{O_2}} = \frac{K_2}{K'_1} + \frac{K_3}{K'_1}\,\frac{Q^{air}_{CO_2}\text{ ferm.}}{Q_{O_2}} \qquad (1a)$$

On voit que c'est l'équation d'une droite dans laquelle le rapport $A_{O_2}/Q_{O_2}$ est fonction linéaire du rapport $Q^{air}_{CO_2}$ ferm./$Q_{O_2}$. Pour déterminer le rapport $K_2/K_3$, il suffit de diviser l'ordonnée à l'origine de la droite par la pente de la droite. L'analyse statistique de 17 adaptations amorcées par le glucose sur maltose, galactose et éthanol montre que les résultats expérimentaux forment une droite et que le coefficient de régression de cette droite est très significatif ($P \ll 0.001$) ; que la meilleure estimée du rapport $K_2/K_3$ est 5.0 et que ses limites sont 4.1 et 6.1 ($P = 0.05$). Il semble donc permis de conclure que l'équation se trouve vérifiée expérimentalement, c'est-à-dire que la vitesse de l'adaptation respiratoire est proportionnelle à l'énergie apportée, que la respiration et la fermentation sont interchangeables, mais qu'un volume d'$O_2$ consommé apporte 5 fois plus d'énergie qu'un volume de $CO_2$ fermentaire dégagé.

Un certain nombre de conséquences importantes découle de cette conclusion. La constante $K_3$ peut être déduite de l'équation générale de la fermentation alcoolique. Si l'on admet que l'énergie utilisable pour la synthèse des hémoprotéines provient des liaisons phosphate riches et que l'on a :

$$C_6H_{12}O_6 + 2\ ADP + 2\ PO_4 = 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP \qquad (2)$$

on trouve que $K_3$ est égal à 0.0446 (mole $\sim$ P/litre $CO_2$) et que le $K_2$ est égal à $0.0446 \times 5 = 0.224$ (mole $\sim$P/litre $CO_2$). Comme on le voit sur la figure 6, $K'_1$ est égal à 0.3 (mole $\sim$P/litre $O_2$). Le bilan global de l'oxydation complète du glucose par la levure devient ainsi :

$$C_6H_{12}O_6 + 6\ O_2 + 30\ ADP + 30\ PO_4 = 6\ CO_2 + 6\ H_2O + 30\ ATP \qquad (3)$$

L'oxydation du glucose apparaît comme étant 15 fois plus efficace énergétiquement que la fermentation et le rapport global $\sim$P/O des phosphorylations oxydatives comme voisin de 2.5. C'est la première fois, à ma connaissance, que l'on a pu établir sur une cellule intacte la relation entre la vitesse d'une synthèse protéique et les vitesses des deux métabolismes, aérobie et anaérobie, démontrer quantitativement leur équivalence et en déduire des conséquences énergétiques. L'accord des données ainsi obtenues *in vivo* avec les prédictions thermodynamiques et avec les mesures directes des phosphorylations oxydatives dans les extraits non-cellulaires est très satisfaisant. En effet, les $-\Delta F_0$ des réactions (*2*) et (*3*) sont 56 kg. cal. et 700 kg. cal. (rapport de 13) et le rapport expérimental $\sim$P/O de l'oxydation du pyruvate *in vitro* est entre 2 et 3 (50).

Connaissant $K'_1$ on peut comparer l'adaptation respiratoire avec la croissance. La levure croît pendant la phase exponentielle avec un temps moyen de génération

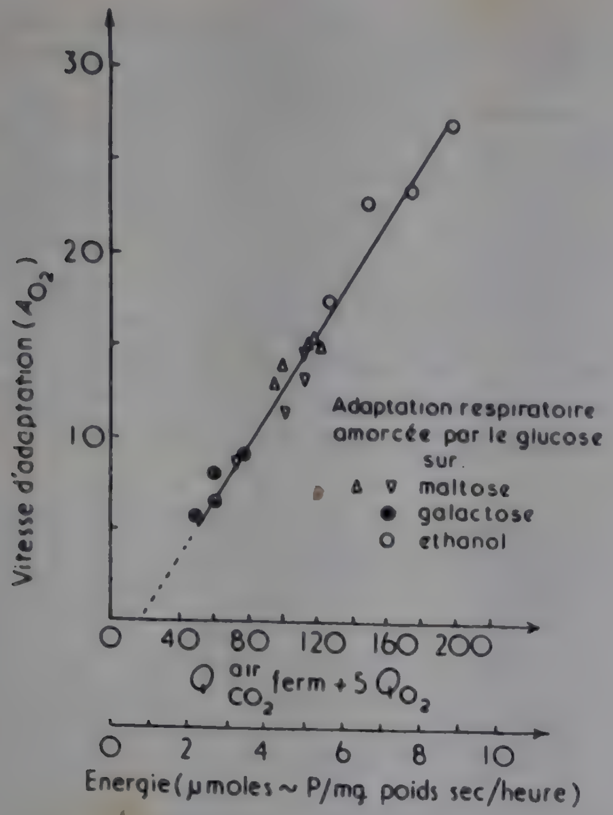


FIG. 6. — Relation entre la vitesse d'adaptation respiratoire et le débit énergétique du métabolisme. Les valeurs des $Q_{O_2}$, $Q^{air}_{CO_2}$ ferm. et $A_{O_2}$ sont mesurées. Le débit énergétique est calculé en admettant $K_3 = 0.0446$ μ-moles ∼ P/mg. poids sec/h.).

de 2.2 h. en anaérobiose et de 1.6 h. en aérobiose. Les intensités métaboliques assurent dans ces conditions un débit de 20 à 30 μ-moles ∼P/mg. poids sec/heure. Autrement dit, à la formation d'un mg. poids sec de protoplasme en deux heures correspond un apport énergétique de l'ordre de 60 μ-moles ∼P. Or, l'adaptation respiratoire, qui a lieu en absence de croissance, exige pour sa vitesse maxima un apport de l'ordre de 20 μ-moles ∼P en deux heures. Elle nous apparaît ainsi comme un processus extrêmement coûteux pour l'économie de la cellule. Notons cependant que $K'_1$ ne représente pas la constante énergétique intrinsèque ($K_1$) de la synthèse d'hémoprotéines respiratoires, mais elle la surestime nécessairement. Des processus métaboliques liés à cette synthèse ont lieu constamment et consomment de l'énergie. La droite de régression qui nous a servi à estimer $K'_1$ (figure 6) montre seulement que l'intensité de ces processus est proportionnelle à la vitesse de l'adaptation respiratoire. Ajoutons en outre que la valeur de 0.3 surestime peut-être légèrement la constante $K'_1$, car les expériences ont été effectuées sur des cellules qui n'ont pas été complètement « rechargées » en matériaux de construction nécessaires à l'adaptation.

### *Matériaux de construction requis pour l'adaptation*

Les expériences décrites jusqu'ici ont été effectuées avec des cellules qui étaient capables de former les enzymes respiratoires en absence d'une source exogène d'azote assimilable. Ces cellules « adaptables » provenaient de la phase stationnaire de la culture anaérobie. L'adaptabilité respiratoire varie fortement au cours de la croissance sans $O_2$. Elle est pratiquement nulle chez les cellules prélevées dans la phase exponentielle de la croissance en milieu contenant $NH_4^+$ comme source d'azote. Or, on sait que la levure contient un important *pool* intracellulaire d'acides aminés libres (51). On sait également que les acides aminés libres jouent un rôle important dans la biosynthèse des protéines (40, 52, 53). On pourrait donc penser que l'absence d'adaptabilité respiratoire des cellules anaérobies provenant de la phase exponentielle est due à l'absence de ce *pool*.

L'expérience représentée par la figure 7 montre qu'il en est bien ainsi. La levure provenant de la phase exponentielle de la croissance anaérobie est incubée anaérobiquement dans l'hydrolysat de caséine. On prélève des échantillons qui, après lavage, sont aérés dans le tampon glucosé. Le prétraitement en absence d'$O_2$ ne change pas la teneur initiale en enzymes respiratoires qui reste nulle. Il augmente par contre la vitesse d'adaptation (figure 7 A). L'évolution de l'adaptabilité respiratoire au cours du prétraitement est discontinue (figure 7 B). On distingue une phase initiale rapide suivie d'une phase beaucoup plus lente. La première a lieu en absence de division cellulaire tandis qu'au cours de la seconde les cellules commencent à se diviser. On peut évidemment penser que la première phase correspond à la recharge du *pool* en acides aminés libres alors que dans la seconde on forme des matériaux de construction plus complexes. Notons que l'ammoniaque est sans action, mais qu'un mélange de 15 acides aminés remplace sensiblement l'hydrolysat de caséine. Il est clair que ce système se prête facilement à une étude détaillée des acides aminés nécessaires à l'adaptation respiratoire générale et aux biosynthèses des hémoprotéines individuelles.

### *Contre-effet Pasteur*

Les résultats suivants fournissent un bon exemple de l'interaction entre le métabolisme fermentaire et respiratoire. La compétition concerne le développement du système hémoprotéique et le métabolisme de ses matériaux de construction.

Depuis les travaux de Meyerhof (6) et pendant une vingtaine d'années l'effet Pasteur a constitué un des problèmes centraux de la biochimie. Il n'est pas question

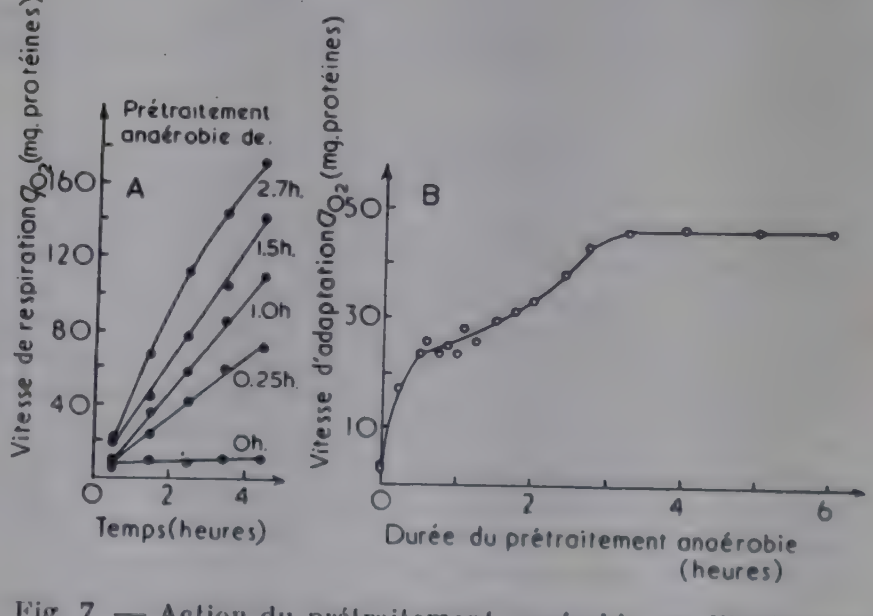


Fig. 7. — Action du prétraitement anaérobie par l'hydrolysat de caséine sur l'adaptabilité respiratoire. Adaptation en glucose 0.015 M.

ici de discuter de ses mécanismes ni même de ses définitions. Il suffit de dire que, quel que soit le sens qu'on lui donne, il consiste en l'inhibition de la fermentation par la respiration (54 à 56). Cette relation a toujours été considérée comme unidirectionnelle. Parmi les quelques centaines de publications qui traitent de l'effet Pasteur, je n'en connais que deux (57, 58) où il est question de la relation inverse. Nous allons voir que la fermentation n'inhibe pas ou très peu la respiration. Par contre, elle inhibe l'adaptation respiratoire.

On a vu comment la fermentation aérobie stimule la vitesse d'adaptation respiratoire en lui fournissant de l'énergie. De l'équation (*1*) on calcule qu'afin d'assurer à une levure ayant un $Q_{O_2}$ initial de 10 sa vitesse d'adaptation maxima ($A_{O_2} = 30$), il faut que le $Q^{\text{air}}_{CO_2}$ ferm. soit initialement de 150 :

$$30 = \frac{0.224 \times 10}{0.3} + \frac{0.0446 \times 150}{0.3}$$

Autrement dit un $Q^{\text{air}}_{CO_2}$ ferm. de 150 est nécessaire et suffisant pour permettre le démarrage de l'adaptation respiratoire à une vitesse maxima.

Supposons qu'une fermentation plus intense soit inhibitrice et que la vitesse de l'adaptation respiratoire soit inversement proportionnelle à l'excédent de la fermentation. On aurait alors pour des $Q^{\text{air}}_{CO_2}$ ferm. supérieurs à 150, la relation suivante :

$$A_{O_2} = 30 \frac{150}{Q^{\text{air}}_{CO_2}\ \text{ferm.}} \qquad (4)$$

La vitesse de la fermentation est fonction de la concentration en glucose. La demi-saturation est atteinte pour la levure intacte par le glucose 8 à 11 $\times 10^{-3}$ M et le $Q_{CO_2}$ suit sensiblement la relation de Henri-Michaelis-Menten (59, 18). En faisant varier la concentration du glucose entre $10^{-3}$ M et 0.2 M on fait varier la vitesse de la fermentation (dont la valeur maxima est de 380) sans influencer la vitesse de la respiration. En effet, la constante de demi-saturation en glucose de cette dernière est inférieure à $5 \times 10^{-4}$ M (60). La figure 8 donne la vitesse de l'adaptation respiratoire en fonction de la concentration en glucose. D'après la relation énergétique (*1*) on doit s'attendre à ce qu'au-dessous de la concentration $6 \times 10^{-3}$ M :

$$\frac{380 \times 0.006}{0.006 + 0.01} = 150$$

les valeurs de $A_{O_2}$ diminuent lorsque le glucose diminue. C'est effectivement le cas. Au-dessus de $6 \times 10^{-3}$ M on doit s'attendre d'après la relation (*4*) à ce que les valeurs de $A_{O_2}$ diminuent lorsque le glucose augmente. La courbe théorique concorde de façon satisfaisante avec les résultats expérimentaux. Le facteur déterminant est la concentration en glucose au début de l'adaptation et non le rapport [glucose]/cellules. Non seulement l'équation (*4*) se trouve ainsi vérifiée, mais en même temps on obtient une vérification expérimentale indépendante de la relation (*1*) de laquelle l'équation (*4*) a été déduite.

L'inhibition par l'excédent de la fermentation concerne tout d'abord la vitesse de l'adaptation respiratoire. Le $Q_{O_2}$ atteint à la septième heure est de 70 ± 10 au lieu de 150 ± 10. La cinétique de l'adaptation dans le temps au lieu d'être linéaire comme en glucose 0.007 M (figure 1) ressemble en glucose 0.2 M à une hyperbole rectangulaire où la vitesse de l'adaptation décroît rapidement. L'extrapolation à l'infini de cette hyperbole fournit cependant un $Q_{O_2}$ voisin de 150. Les biosynthèses des hémoprotéines individuelles sont inhibées de façon inégale. La fermentation excédentaire ralentit surtout la formation de la succino-cytochrome *c* réductase, influence moyennement la formation de la lactico-cytochrome *c* réductase et du cytochrome *c*, tandis que la biosynthèse de la cytochrome *c* oxydase y est relativement peu sensible.

Quels que soient les détails de son mécanisme, la conséquence principale de l'inhibition par la fermentation est l'empêchement de l'effet Pasteur. Bien que ceci paraisse constituer une lapalissade, l'effet Pasteur, qui consiste en la diminution de la vitesse de fermentation par le métabolisme respiratoire, n'est possible qu'en présence de ce dernier, donc en présence des hémoprotéines (18). En ralentissant la biosynthèse des enzymes respiratoires, le métabolisme anoxybiotique contrecarre l'établissement du métabolisme respiratoire inhibiteur de la fermentation. C'est le contre-effet Pasteur.

Le fait le plus remarquable est l'ajustement étroit du métabolisme de la levure. Le système régulateur global est composé de trois relations interdépendantes :

— celle de Pasteur-Meyerhof,

$$\left(Q^{N_2}_{CO_2} - Q^{\text{air}}_{CO_2}\ \text{ferm.}\right) / Q_{O_2} = 2 \text{ à } 3.$$

— celle des phosphorylations permettant l'adaptation respiratoire

$$A_{O_2} = 0.75\ Q_{O_2} + 0.15\ Q^{\text{air}}_{CO_2}\ \text{ferm.}$$

— celle de la fermentation inhibant l'adaptation respiratoire

$$A_{O_2} = 4500/Q^{\text{air}}_{CO_2}\ \text{ferm.}$$

et de trois constantes, $Q^{N_2}_{CO_2}$ max = 380, $Q_{O_2}$ max = 150 et $A_{O_2}$ max = 30. Le débit critique de la fermentation qui fournit les phosphorylations nécessaires et suffisantes à l'adaptation respiratoire maxima et sépare de ce fait les deux dernières relations est de l'ordre de 150 $\times$ 0.0446 = 7 μ-mole ~P/mg. poids sec/h. La compétition dont rend compte la première relation concerne vraisemblablement les phosphorylations (61 à 63). Il pourrait en être de même pour le contre-effet Pasteur, la fermentation excédentaire provoquant une carence en phosphate inorganique ou en accepteurs de phosphate comme l'ADP. Ce qui est certain c'est l'interaction entre l'inhibition provoquée par la fermentation excédentaire et l'ampleur du *pool* des acides aminés. En effet, les cellules rechargées complètement dans l'hydrolysat de caséine sont beaucoup moins sensibles à cette inhibition que les cellules rechargées partiellement (tableau II).

Il est probable que l'inhibition de l'adaptation respiratoire peut servir d'explication à quelques observations dont certaines sont déjà assez anciennes. Elle explique la diminution de la consommation d'$O_2$ par addition de glucose chez une levure de bière (qui s'adapte) et

TABLEAU II

*Intensité du contre-effet Pasteur en fonction de la recharge en acides aminés*

| Durée du prétraitement anaérobie dans l'hydrolysat de caséine (min.) | Adaptation respiratoire | | |
|---|---|---|---|
| | air $Q_{CO_2}$ ferm. | $A_{O_2}$ | Inhibition (%) |
| 45 | 161 | 30.3 | — |
| | 276 | 16.4 | 46 |
| | 368 | 10.3 | 66 |
| 210 | 180 | 34.5 | — |
| | 265 | 29.2 | 15 |
| | 381 | 22.7 | 34 |

l'absence de diminution chez une levure de boulangerie, déjà adaptée (57). On sait également que la levure, au cours de la croissance aérobie en présence de fortes concentrations de glucose le dégrade presque entièrement en alcool (64, 65), sa respiration est faible (64, 66) et sa teneur en cytochromes diminuée (66). La respiration (2, 6, 66) et les cytochromes (66, 67) augmentent à la fin de la croissance. Or, pour une levure en train de proliférer, ce qui compte autant que le fonctionnement c'est la formation des enzymes respiratoires. Nous avons vu que cette formation est inhibée par des fortes concentrations en glucose, ce qui permet de rendre compte, il me semble, de ces observations.

La biosynthèse d'un grand nombre d'enzymes chez des microorganismes assez divers est inhibée par le glucose (69). Cette inhibition constitue la base du phénomène de la « diauxie » (68). Il est peu probable que le même mécanisme opère dans tous les cas. On peut cependant garder présent à l'esprit le cas de l'adaptation respiratoire chez la levure en tant qu'exemple d'inhibition causée par un métabolisme trop intense par rapport aux besoins énergétiques des biosynthèses.

## ÉTAPES DE L'ADAPTATION RESPIRATOIRE

L'adaptation respiratoire se poursuit à vitesse constante sur la presque totalité de son parcours (figure 1). On serait tenté de considérer cette cinétique simple comme l'expression d'un processus qui, à chaque moment de sa progression, est semblable à lui-même. Il est cependant possible de mettre en évidence des étapes différentes au cours du développement des hémoprotéines respiratoires.

### *Etape critique*

Lorsque l'adaptation respiratoire est limitée par le débit énergétique, que ce soit sur éthanol (figure 4 B) ou au moyen de la double adaptation (figure 5), l'accroissement du $Q_{O_2}$ devient linéaire à partir d'un certain moment. Ce moment peut suivre immédiatement le début de l'aération (figure 4 B, figure 5, maltose) ou en être éloigné de plusieurs heures (figure 5, galactose). Or, l'apport d'énergie s'accroît au fur et à mesure que l'adaptation progresse. On s'attendrait donc à ce que la vitesse d'adaptation augmente à partir du moment de l'amorçage au lieu de rester constante. Tout se passe comme si l'accroissement du débit énergétique délivré postérieurement au moment du déclenchement était inefficace.

Lorsque l'adaptation respiratoire est limitée par la teneur initiale en matériaux de construction, l'accroissement du $Q_{O_2}$ est également constante (figure 7 A). La preuve de la diminution du *pool* des acides aminés n'a pas été apportée dans le cas de la biosynthèse des hémoprotéines. On sait cependant que le *pool* diminue au cours de la formation induite de la maltozymase (40). On pourrait donc penser que la vitesse d'adaptation diminue au fur et à mesure de la formation des enzymes respiratoires. Or, il n'en est rien.

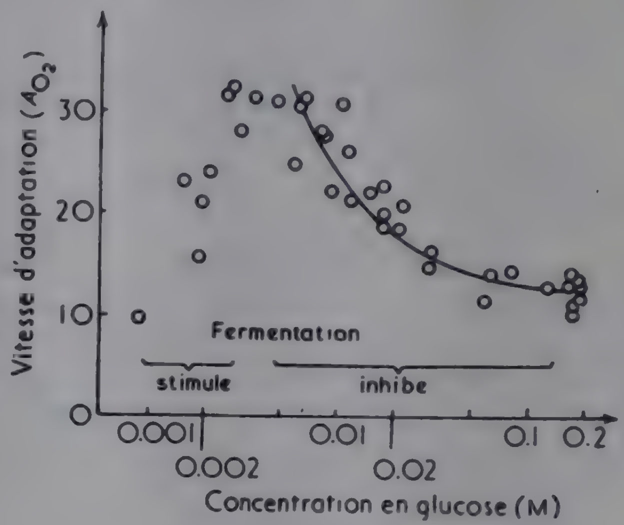


Fig. 8. — Vitesse de l'adaptation respiratoire en fonction de la concentration initiale en glucose. La courbe théorique est calculée selon l'équation (4a) :

$$A_{O_2} = 30 \frac{150\,([G] + 0.01)}{380\,[G]}$$

Une fermentation initiale trop intense inhibe l'adaptation respiratoire. L'inhibition est irréversible car une diminution ultérieure du $Q^{air}_{CO_2}$ ferm. n'augmente pas la vitesse de la formation des hémoprotéines. Cette diminution peut être réalisée de deux manières avec le même résultat : soit en diminuant, par exemple, une heure après le début de l'adaptation, la concentration en glucose, soit spontanément comme conséquence de l'entrée en jeu de l'effet Pasteur-Meyerhof. Notons d'ailleurs que cet effet ne se manifeste qu'avec un retard considérable par rapport à l'accroissement du $Q_{O_2}$.

Il semble donc permis de conclure qu'une « étape critique » est à l'origine du développement des hémoprotéines. Sa durée est inférieure à 1 h., donc inférieure à un cinquième de la durée totale de l'adaptation respiratoire. Au cours de cette étape critique, la vitesse avec laquelle les enzymes respiratoires seront formés ultérieurement est déterminée par l'interaction entre l'apport d'énergie et les matériaux de construction disponibles. On serait tenté de voir dans cette étape critique une étape de l'induction par l'$O_2$ des biosynthèses hémoprotéiques

analogue à la phase latente des autres biosynthèses induites des enzymes (70).

Bien que l'adaptation respiratoire possède une certaine irréversibilité à partir de l'étape critique, cette irréversibilité n'est pas totale. L'absence de l'inducteur ($O_2$), les analogues des acides aminés, arrêtent l'adaptation comme le fait également l'addition du glucose qui provoque une fermentation excédentaire (figure 9). Après le brusque arrêt d'une heure environ, l'adaptation reprend spontanément. Il est important de noter que quel que soit le moment entre 0 et 4 h. du déclenchement du contre-effet Pasteur, le niveau d'adaptation atteint à la dixième heure est le même. On verra qu'un phénomène analogue se produit avec le benzimidazole.

Le benzimidazole inhibe l'adaptation respiratoire. L'inhibiteur absorbé par la cellule peut en être enlevé presque immédiatement par un simple changement du pH extracellulaire. En effet, seule la molécule non chargée

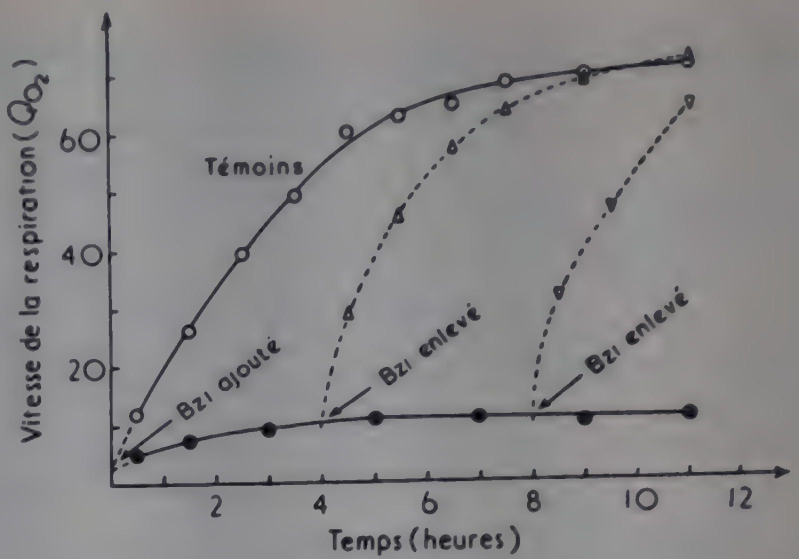


Fig. 10. — Accélération temporaire de la vitesse d'adaptation après la levée de l'inhibition provoquée par le benzimidazole (Bzi). Adaptation en glucose 0.17 M ; Bzi présent : cercles noirs ; Bzi absent : cercles clairs.

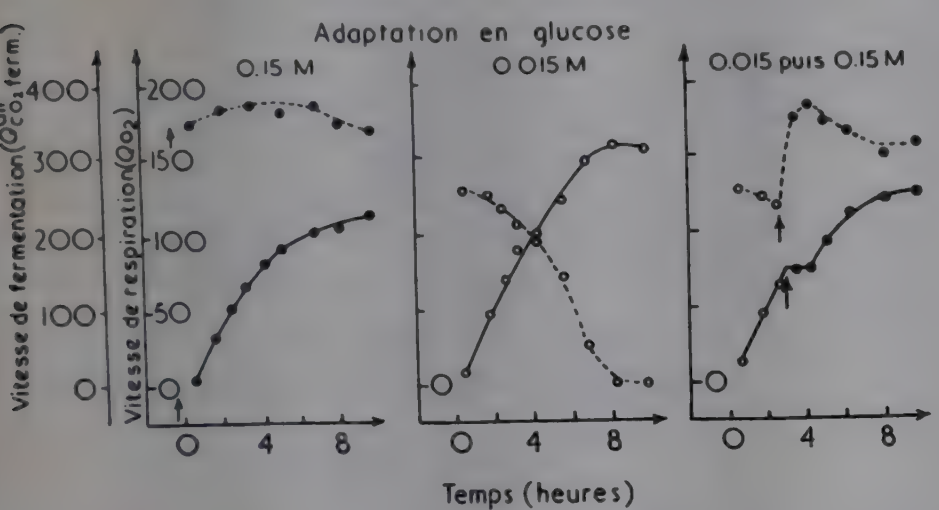


Fig. 9. — Contre-effet Pasteur provoqué au début et au milieu de l'adaptation respiratoire. Les flèches indiquent le moment de l'addition du glucose 0.15 M. Trait plein : respiration ; trait pointillé : fermentation.

pénètre dans la levure (71, 72). Le mode d'action du benzimidazole n'est pas bien connu. Son action sur l'assimilation des glucides ressemble à celles du 2,4-dinitrophénol et du $NaN_3$ (72). Les résultats selon lesquels il agirait en tant qu'antimétabolite des purines (73) ont vraisemblablement été dus à un artefact (71). Lorsqu'on enlève le benzimidazole, l'adaptation reprend (figure 10). Cependant, l'adaptation ainsi restaurée se produit avec une vitesse initiale plus grande que la vitesse initiale du témoin. Un accélérateur des biosynthèses s'est donc accumulé pendant que la formation des hémoprotéines a été supprimée. Cet accélérateur possède la propriété de permettre un $A_{O_2}$ de plus de 30 en présence d'une fermentation très intense. Autrement dit, il rend les cellules temporairement insensibles au contre-effet Pasteur. Néanmoins le $Q_{O_2}$ finalement atteint est partout le même.

TABLEAU III

*Inhibition de l'adaptation respiratoire par la 2,6-diaminopurine*

| Levure | Adaptation | $Q_{O_2}$ à 0 h. | $Q_{O_2}$ à 7 h. | Inhibition de l'adaptation (%) |
|---|---|---|---|---|
| A | témoin | <5 | 77.5 | — |
| | + DAP 3.5 mM | » | 80.1 | 0 |
| B | témoin | » | 36.5 | — |
| | + DAP 3.5 mM | » | 19.8 | 46 à 53 |
| | + DAP 3.5 mM + adenine 3.3 mM | » | 35.6 | 2 à 3 |
| C | témoin | » | 15.8 | — |
| | + DAP 3.5 mM | » | 5.5 | 65 à 95 |
| | + DAP 1.4 mM | » | 11.5 | 27 à 40 |

La levure A est rechargée complètement en acides aminés. Les levures B et C sont rechargées partiellement. Adaptation en glucose 0.17 M.

Il ne dépend pas de l'histoire préalable de l'adaptation, de la suppression de l'inhibition causée par le benzimidazole.

Il est difficile d'interpréter à l'heure actuelle l'ensemble de ces résultats. Il semble cependant probable que nous rencontrons ici le problème essentiel du développement des hémoprotéines, le problème du système formateur des enzymes respiratoires. Les modifications telles qu'on les voit sur les figures 9 et 10 ne seraient peut-être pas autre chose que des modifications et des reconstitutions de « l'organisateur » des biosynthèses hémoprotéiques (48, 70) dont la vie moyenne correspondrait à la durée de l'étape critique et à la durée de l'inhibition par la compétition fermentaire. On sait le rôle probable des nucléotides dans la synthèse protéique (74). Il est intéressant de constater que les analogues des purines ou pyrimidines (2,6 diaminopurine, les azapurines, 6-méthyluracil, 5 Bruracil, diazouracil, 2-thiouracil) n'ont aucune action sur l'adaptation respiratoire normale. Par contre ils ont une action manifeste lorsque le développement des hémoprotéines a été dérangé pendant l'étape critique (tableau III). Les nucléotides jouent donc vraisemblablement un rôle dans l'organisation de la biosynthèse des hémoprotéines.

### *Etape héréditaire*

L'adaptation respiratoire dans son ensemble n'est pas un phénomène héréditaire. Lorsque la levure pousse en absence d'$O_2$, la biosynthèse des hémoprotéines respiratoires n'a pas lieu, mais la capacité d'effectuer cette synthèse est maintenue même après plusieurs centaines de générations cellulaires.

Les recherches d'Ephrussi et de ses collaborateurs (75) ont montré que les acridines induisent chez la levure une mutation spécifique. Elle est due à la perte ou à la mutation d'un facteur cytoplasmique « autoreproductible » responsable de la transmission héréditaire de certaines hémoprotéines (cytochrome *c* oxydase, succino-cytochrome *c* réductase, DPNH cytochrome *c* réductase (18). Par contre, ce facteur n'est pas responsable de la transmission héréditaire des autres enzymes respiratoires comme le cytochrome *c*, la lacticocytochrome *c* réductase, la catalase, la cytochrome *c* peroxydase (18, 22, 23). Les mêmes acridines, et les seules qui induisent la mutation (76), arrêtent l'adaptation respiratoire en bloquant sélectivement la biosynthèse de la cytochrome *c* oxydase (19). Elles n'affectent pas l'activité de cet enzyme. L'inhibition de l'adaptation précède l'induction de la mutation. On peut supprimer complètement la biosynthèse de la cytochrome *c* oxydase sans que les mutants apparaissent. Quelques heures plus tard, la majorité des cellules sont mutées et incapables à jamais de former cette hémoprotéine. Il existe donc une étape, ou une réaction, qui est commune au développement non-héréditaire et à la transmission héréditaire des enzymes respiratoires.

L'étude de l'adaptation respiratoire chez la levure, a permis d'aborder un certain nombre de problèmes concernant les oxydations cellulaires, l'énergétique du métabolisme aérobie et anaérobie, les régulations intracellulaires, la biosynthèse des protéines et l'hérédité cellulaire. Le développement des hémoprotéines induit par l'$O_2$ apparaît comme un processus presque aussi complexe que le développement d'un virus ou la croissance de la cellule. Quelques relations ont pu, néanmoins, être dégagées sous une forme assez simple.

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# Metalloflavoproteins and electron transport

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(*Received* 4 *April* 1955)

Electron transport, at least in aerobic organisms and tissues, usually implies the use of oxygen as the final oxidant in a chain of oxido-reduction enzymes which have the substrate as the original reductant. This chain may only consist of one link ; in that case we speak of oxidation by a true oxidase. More frequently, however, at least two additional links are involved : primary dehydrogenation is usually catalyzed by a pyridine-nucleotide requiring dehydrogenase, while the final interaction with oxygen is mediated by the cytochrome system.

It has long been recognized that those enzymes which contain the nucleotide derivatives of riboflavin as their prosthetic groups, *i.e.* the flavoproteins, play a pivotal role in this process of linking the substrate to oxygen. By virtue of their structure and thus explicitly their oxido-reduction potential flavoproteins are ideally fitted to be easily reducible by materials of $E_0'$ of —0.100 volts or so and below and oxidizable by those with potentials above this value. This holds true for riboflavin itself and was commonly accepted for flavoproteins as well.

To be sure not all was serene in the flavin field. Some flavoproteins gave strong indication of possessing additional components and it has long been known that there appears to be a great deal of variability of the relative ease of the reoxidation of reduced flavoproteins by various electron acceptors, and that only a few flavoproteins, presumably with rather special properties are capable of interaction with the cytochrome system (1). But only during the past two years has it become apparent that there exists a large number of flavoproteins which contain a metal as well as part of their prosthetic group, that the members of this class are those flavoproteins which are capable of being oxidized by a cytochrome and thus participate in electron transport, and that there exists a direct causal relationship between those two facts.

The discovery of metalloflavoproteins, as is true of almost all such findings, took place almost simultaneously in several laboratories and was, at least in our case, serendipital to quite a different line of investigation : in the course of our investigations on the enzymes conerned

with the oxidation of fatty acids (2) we found ourselves with a dark green flavoprotein on our hands, somewhat the color of a creme de menthe frappee (3, 4). The quantitative counterpart of this color was a strong band around 680 mμ (figure 1) which on combination with the usual flavoprotein spectrum could account for the visual observations. All three bands were affected by the presence of the substrate (*i.e.* butyryl CoA), and this reduction in intensity could be counteracted by the addition of the product (crotonyl-CoA) or of appropriate

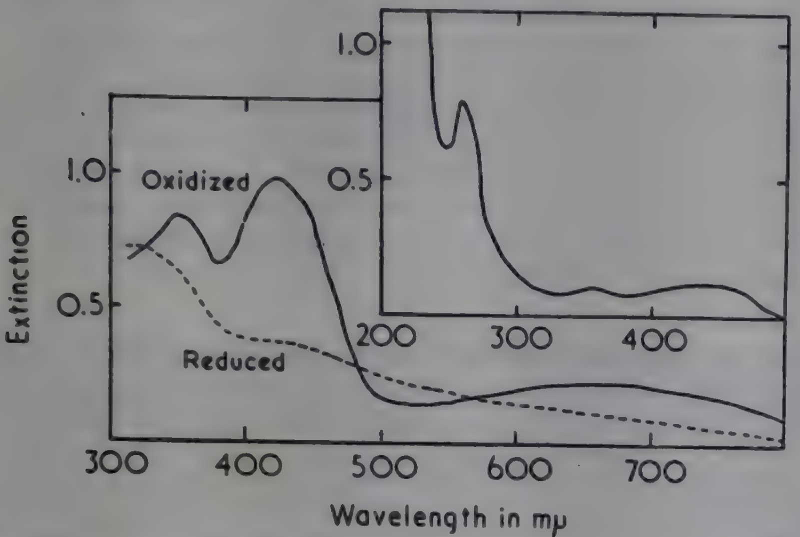



Fig. 1. — Absorption spectrum of butyryl-CoA dehydrogenase. Spectrum of electrophoretically purified enzyme of specific activity 0.98. Data plotted as relative extinctions, assuming an extinction equal to unity at 435 mμ. Visible spectrum shown on large graph and ultraviolet range in insert. The 'reduced' spectrum was obtained by the addition of 50 mμ-moles of butyryl-CoA to the cuvette containing the preparation just described. From Mahler (4).

oxidizing agents (table I). The presence of copper to account for this spectral anomaly appeared strongly implicated and indeed quantitative copper determination unambigously proved the presence of the metal as an integral part of the enzyme and as standing in definite

Table I

*Flavin and copper content of butyryl-CoA dehydrogenase*

| | Preparation 6 | Preparation 7-1 |
|---|---|---|
| Enzyme, units/g. | 250 | 980 |
| Flavin, μ-moles/g. | 5.6 | 27 |
| Copper, μ-atoms/g. | 12.3 | 54 |
| Atoms copper/moles flavin | 2.2 | 2.0 |
| m-Atoms copper/unit | 49 | 55 |

proportion to the more conventional riboflavin portion of the prosthetic group which in turn could be characterized as FAD.

Establishing a role for the copper was a more elusive pursuit. First of all we removed the metal in the classical manner by dialysis *vs.* cyanide. This led to almost complete removal of the long wave length band and a change in appearance from green to yellow (figure 2). Reduction of the enzyme bound flavin by the substrate proceeded with undiminished vigor, reduction of dyes

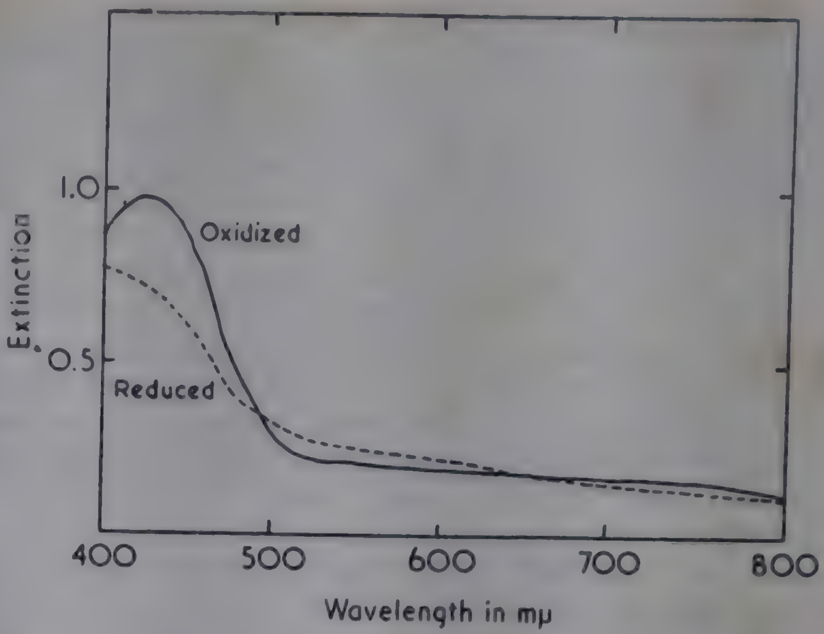



Fig. 2. — Spectrum of $E_{CN}$. Data plotted in a manner analogous to that of figure 1; reduced spectrum obtained on addition of butyryl-CoA. From Mahler (4).

and other electron acceptors, such as it was, appeared unimpaired and we were just about to run out of ideas when we decided to make one more try (we remembered that the enzyme catalyzed some sort of reaction of substrate with cytochrome *c*, figure 3). Not enough to be of any importance physiologically we thought, nor even to be of any use analytically. But at least there was some interaction. The cyanide-treated enzyme on

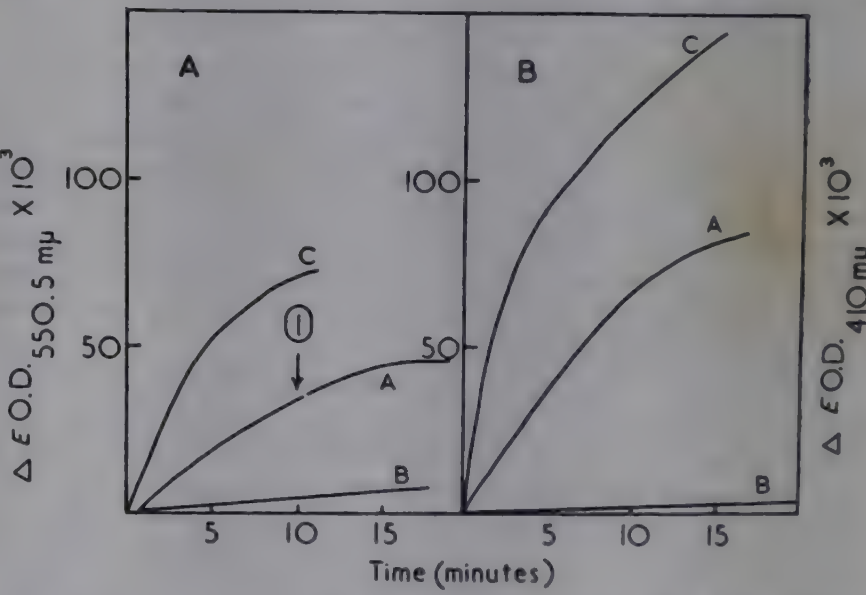



Fig. 3. — Reduction of ferricytochrome *c* and ferricyanide by butyryl-CoA and dehydrogenase. In **A** the cytochrome *c* assay was used. 1.0 mg. of cytochrome *c* 10 μ-moles of Tris buffer, pH 7.0, and 0.067 μ-moles of butyryl-CoA in a total volume of 0.95 ml. are placed in a cuvette and the optical density determined at 550.5 mμ for several minutes. When no further change occurs, the enzyme solution (0.05 ml.) is added and the optical density measured at the times shown. Curve A, $E_{CN}$ (50 μg.), 0.5 μ-moles of $CuSO_4$ added at Point 1. Curve B, $E_{CN}$ (50 μg.) incubated for 10 minutes at 38° C. in the presence of the buffer and 0.5 μ-moles of $CuSO_4$ (butyryl-CoA omitted). Curve C determined as follows : $E_{CN}$ (50 μg.) preincubated as in Curve B, added to dye and substrate at O time. Solid line, untreated (not split by cyanide) enzyme (50 μg.). In B, all details similar to those in *A*, except that 0.5 μ-moles of ferricyanide was used as an acceptor and the decrease in optical density at 410 mμ was used as a measure of the reaction. From Mahler (4).

the other hand was almost completely inert. To our great joy preincubation of the enzyme with copper, however, restored the activity completely.

Now I have spent a considerable amount of time discussing butyryl-CoA dehydrogenase because for us it provided the door into the exciting edifice of the metalloflavoproteins, which in turn proves to be but an anteroom of a far more awe-inspiring structure beyond, that of the true electron transport system. The observations that we made with this enzyme and some of the conclusions that we were able to draw from them proved to be applicable to all the large number of metalloflavoprotein enzymes that we were able to study subsequently. But we were very lucky : our samples of butyryl-CoA dehydrogenase were just pure enough as to give us completely unambigous answers concerning its functions, its composition and its structure, but they were not as pure as some subsequent samples of the same enzyme that Drs. Beinert and Crane studied, and which led them to the discovery of the electron transferring flavoprotein (5).

Others got to the field of metalloflavoproteins by different approaches : Albert (6) suggested that on the basis of his experiments which showed that riboflavin was capable of forming chelates with heavy metals that flavoproteins, most notably xanthine oxidase might well be re-examined for possible metal content. While his paper was still in press, De Renzo (7) at the Lederle Laboratories proved by excellent analytical work that the hitherto unidentified nutritional XO factor (8), one required for the elaboration of xanthine oxidase by rats was simply inorganic molybdenum. This announcement was followed within a matter of weeks by the demonstration that milk xanthine oxidase did indeed contain molybdenum as an essential part of the enzyme, and that the metal followed along with the flavin during purification. A group working with De Renzo at Lederle (9), one with Totter at Oak Ridge (10), ourselves (11) and Reichert and Westerfeld (12), the original discoverers of the XO factor all were able to arrive at this result independently.

Nason and Nicholas (13) at the McCollum Pratt Institute meanwhile discovered that molybdenum was an essential growth factor for *Neurospora crassa*, that it was required for the elaboration of nitrate reductase activity in the mold and that it appeared to form an essential link in the electron transport scheme between flavin and nitrate. Finally Harrison (14) showed that fumaric hydrogenase, that strange unidirectional flavoprotein of Fischer's (15) was activated by ferrous ions.

Since then a large number of enzymes have been shown to fall into the metalloflavoprotein class. Figure 4 shows a generalized scheme of electron transport in which I have attempted to classify flavoprotein enzymes more or less arbitrarily into three classes : A — those enzymes whose substrate is in turn the coenzyme of a previous redox enzyme (*i.e.* a pyridine nucleotide) and whose acceptor is probably a member of the cytochrome system, or some other oxidant differing from molecular oxygen. B — are those enzymes which oxidize substrate without the intervention of pyridine nucleotide, which may or may not be able to function aerobically, but which are capable of reducing cytochrome *c* or some

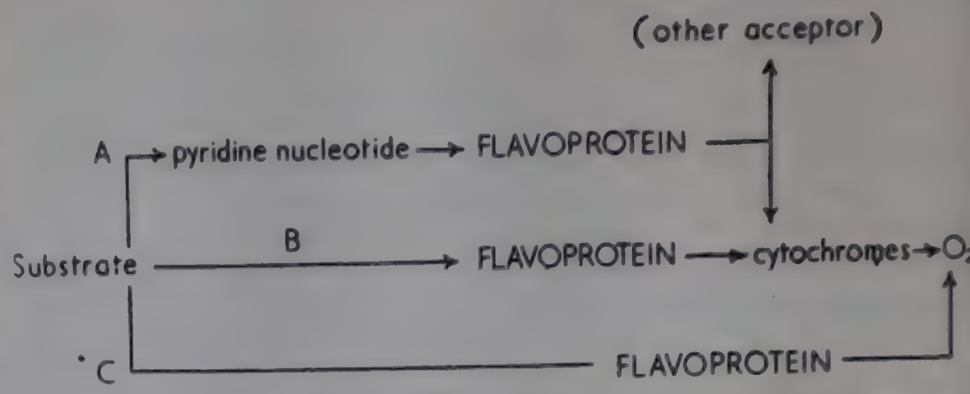



FIG. 4. — Role of flavoproteins in electron transport. A : DPNH-oxidase; DPNH-cytochrome reductase; TPNH-cytochrome reductase; xanthine oxidase; nitrate, nitrite, hydroxylamine reductases; diaphorase; old, new yellow enzymes; quinone reductases. B : Lactic oxidase; aldehyde oxidase; xanthine oxidase; butyryl-CoA dehydrogenase; hydrogenase; succinic dehydrogenase; sulfite oxidase. C : Amino-acid oxidases; glucose oxidase; amine oxidases (?).

other cytochrome, while I have placed the true oxidases in class C. Where do we look for metalloflavoproteins ? The safest way of approaching this question is to determine which enzymes definitely are not. It turns out that those in class C, do not possess any metal component. Neither the highly purified L-amino acid oxidase from water moccasin venom (16), nor a highly purified D-amino acid oxidase isolated in the conjugated form by Dr. Fairhurst nor glucose oxidase (18) show the least trace of metal involvement by spectroscopic analysis. Nor are they capable of interacting with certain electron acceptors of a rather special kind.

The enzymes in the other two classes are definitely metalloflavoproteins or at least have been associated with metal-containing components prior to their isolation and purification. Table II classifies the numerous isolated metalloflavoprotein enzymes according to the known metal component. It can be seen that although a very large variety of enzymes are involved, the variety of metals is much smaller. Only three have been established with certainty so far : copper, iron and molybdenum. What then is the role of the

TABLE II

*Metals in metalloflavoproteins*

| Metal | Enzyme |
|---|---|
| Copper | Butyryl-CoA dehydrogenase |
| Iron | DPNH-cytochrome reductase<br>DPNH oxidase<br>Succinic dehydrogenase (19)<br>TPNH-cytochrome reductase (20)<br>Acyl-CoA dehydrogenase ? |
| Molybdenum | Xanthine oxidase<br>Aldehyde oxidase<br>Nitrate reductase<br>Hydrogenase (28) |
| Unknown | Nitrite reductase (21)<br>Hydroxylamine reductase (21)<br>Sulfite oxidase (22) |

metals ? Experiment has shown that electron acceptors can be separated into two different classes (figure 5). Whenever we have been able to separate the metal from the enzyme, or to inhibit it by treatment with suitable complexing or chelating agents, such an elimination of the metal has had no effect on the efficiency of interaction of the flavoprotein in question with electron acceptors of class II. Class II, as you can see includes

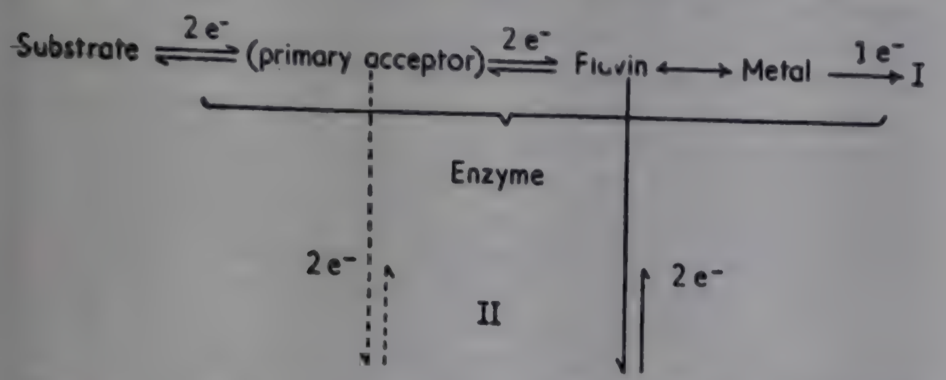



FIG. 5. — Mode of action of metalloflavoproteins. Acceptors of class I : cytochrome *c*; enzyme-bound cytochrome ('*b*'); aldehyde oxidase, yeast lactic oxidase (?); nitrate, nitrite, hydroxylamine; ferricyanide; methyl, benzyl viologens.
Acceptors of class II : $O_2$; redox dyes ($2\ e^-$); quinones.

oxygen as its physiologically most important member and almost all the dyes and redox indicators usually employed in work with oxidation enzymes. An absolute requirement for metals is associated with oxidation by the electron acceptors of group I. It includes cytochrome *c*, and probably the other cytochromes as well, it includes 'built-in' cytochrome components, a concept which I shall discuss shortly, it includes nitrate, nitrite (21) and probably hydroxylamine (21), nitrogen compounds which probably have to be reduced in one-electron steps, and the one-electron acceptors ferricyanide and methyl- and benzyl- viologen (23). What peculiarity is there about acceptors in this group which would necessitate a completely different mechanism ?

Let us confine our attention to the cytochromes, to ferricyanide, and to the viologens. Inspection of their formulae (figure 6) makes it quite clear that these

1 $\quad [Fe(CN)_6]^{-3} + 1e^- \rightleftharpoons [Fe(CN)_6]^{-4}$

2 $\quad$ Cytochrome $c(Fe^{+++}) + 1e^- \rightleftharpoons$ Cytochrome $c(Fe^{++})$

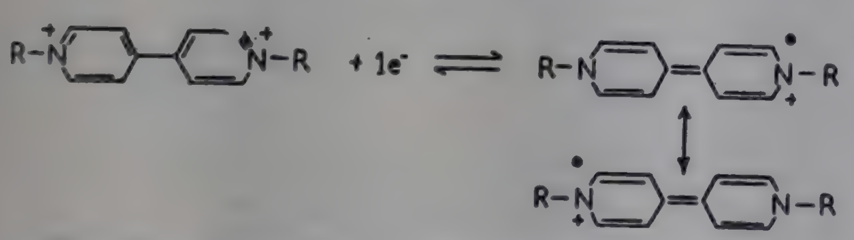
3. 

FIG. 6. — Reduction of one-electron acceptors.

coumpounds are true electron acceptors, *i.e.* they can be reduced only by virtue of acquiring an electron and only one electron and not by interaction with a hydrogen atom or a hydride ion with its associated electrons. Now pyridine nucleotides are reduced and oxidized precisely by this latter path as the brilliant work of Westheimer, Vennesland and their group at Chicago (24) has demonstrated (figure 7). Structural and kinetic similarities make it appear likely that flavoprotein as well are reduced by this mechanism, and that probably their re-oxidation by acceptors of class II also proceeds

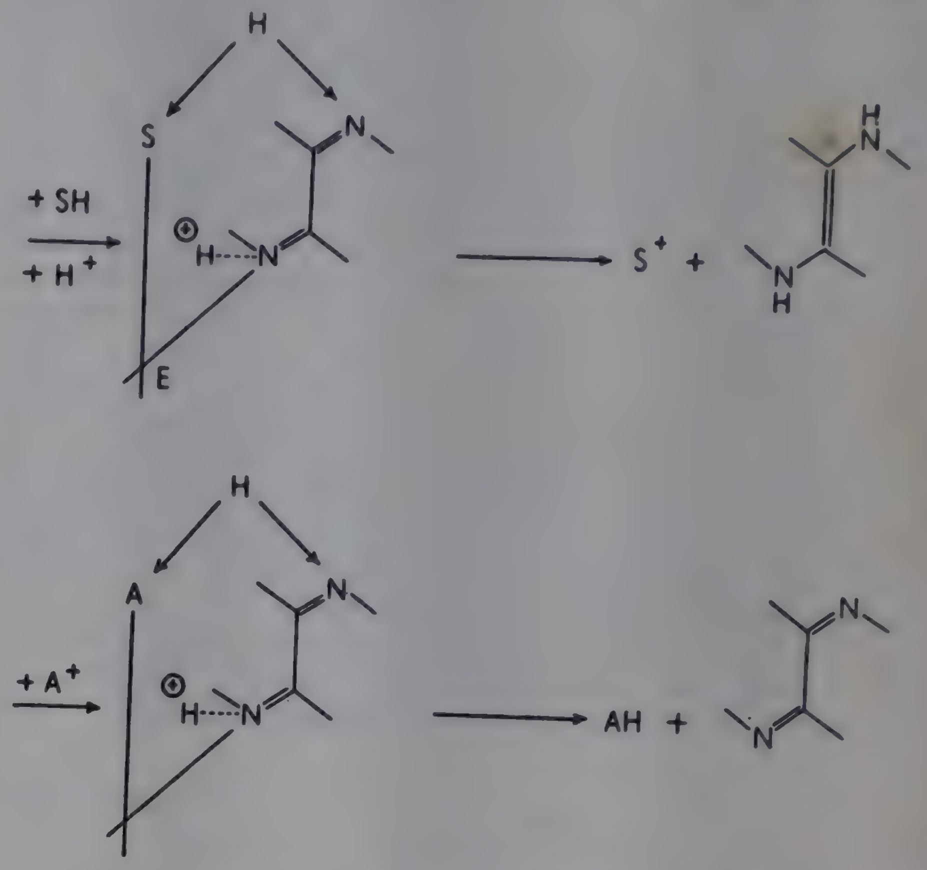



FIG. 7. — Transition states in flavoprotein catalysis.

by this path (figure 8). No such possibility exists for the oxidizing agents of class I. The transition state either involves a flavin semiquone, or a kinetically prohibitive ternary complex. Thus the reduction of the flavoprotein and its reoxidation must here proceed by two different mechanisms. The metal makes this possible : *a*) it will stabilize the semiquinone intermediate of the flavoprotein by introducing additional resonance possibilities (figure 8), and thus increase the

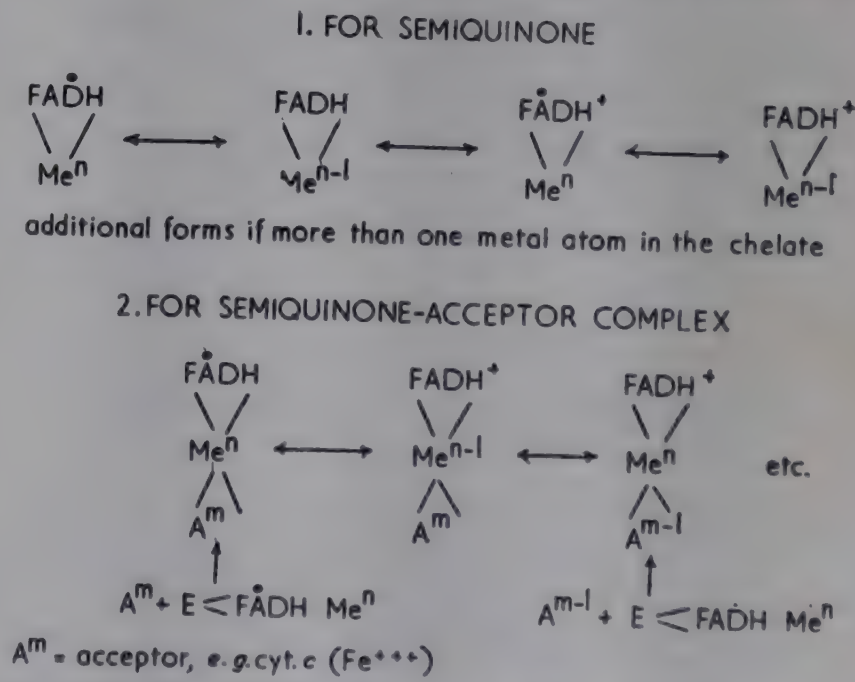



FIG. 8. — Resonance forms of flavin semiquinones.

life-time and the stability of this intermediate, formed after the removal of one electron, long enough to make possible effective transfer of the second electron to the second molecule of acceptor; *b*) by providing a direct link between flavin and two molecules of acceptor within one complex the two-electron donor one-electron acceptor problem becomes an intramolecular rather than an intermolecular one. No three-body collision is necessary and the process of electron transport between two, or even worse three, molecules simply becomes one of electron transfer within the same molecule; *c*) it is apparent that we can write additional resonance forms involving the metal not only for the metalloflavin semiquinone intermediate but also for the reduced flavoprotein itself as well as for the complexes involving reduced metalloflavoprotein and acceptor. Two mechanisms, both giving rise to a considerable decrease in activation energy over any not involving metal might be envisaged. One involves an actual semiquinone as an intermediate, and is shown as part 2 of figure 8. The second (figure 9) does not postulate a flavin semiquinone even as a transitory, real intermediate but is based simply on a direct intramolecular electron transfer from one part of the molecule, (*i.e.* the flavin through the metal to the acceptor) all within the same resonating system, followed by dissociation of the reduced acceptor molecules. That this latter process will be one requiring very little activation energy is self-evident. Both processes will be associated with low activation energy and be of high efficiency. For it has been shown that in any process in which the reacting species shows a great many structural similarities to the activated one is one that will proceed with great ease. This is precisely the situation which we meet with here. Thanks to the presence of the metal similar structures, both involving unpaired electrons can be written not only for the intermediate (and the transition state) but for the ground state of the reacting molecule as well. This mechanism of electron transfer between two systems (as distinct from transport) within the framework of a complex with the metal ion providing the link which makes possible this transmission is the biochemical counterpart of one proposed by Taube (25). He has studied certain inorganic reactions which may be envisaged to proceed by means of a complex in which an anion (*e.g.* chloride) provides the link and makes possible transfer of electrons between metal ions.

What experimental evidence is there to back up these postulates? That one-electron acceptors are actually bound through the metal has been demonstrated in two ways.

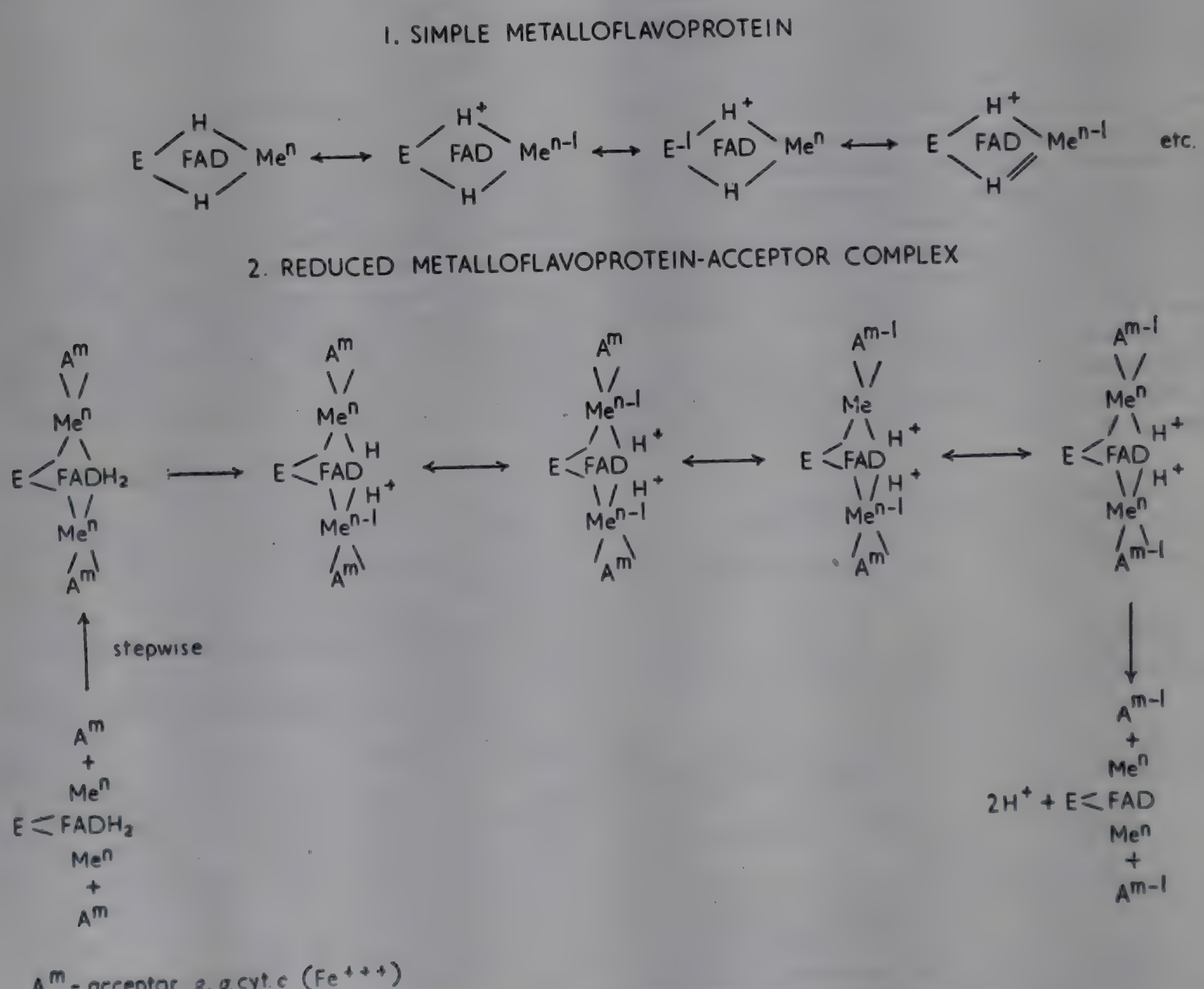



**Fig. 9. — Semiquinoid resonance forms of reduced flavin.**

DPNH cytochrome reductase, a ferroflavoprotein (35) is inhibited very effectively by iron-chelating anions such as pyrophosphate or citrate (figure 10). Now this inhibition is overcome in a competitive manner by increasing concentrations of one-electron acceptor (for instance cytochrome *c*). If we grant that the inhibition is due to interaction with the iron, then it follows that the reversal of the inhibition by the cytochrome must also be due to binding at the metallic site.

There is an ever-increasing body of evidence that some metalloflavoproteins themselves may bear the same relationship to the native electron transport enzymes as do certain metal-free flavoproteins to their metalloflavoproteins counterparts. In other words not only has it been possible to isolate certain metalloflavoproteins which contain a cytochrome component as part of the molecule, but in some instances whole hierarchies of

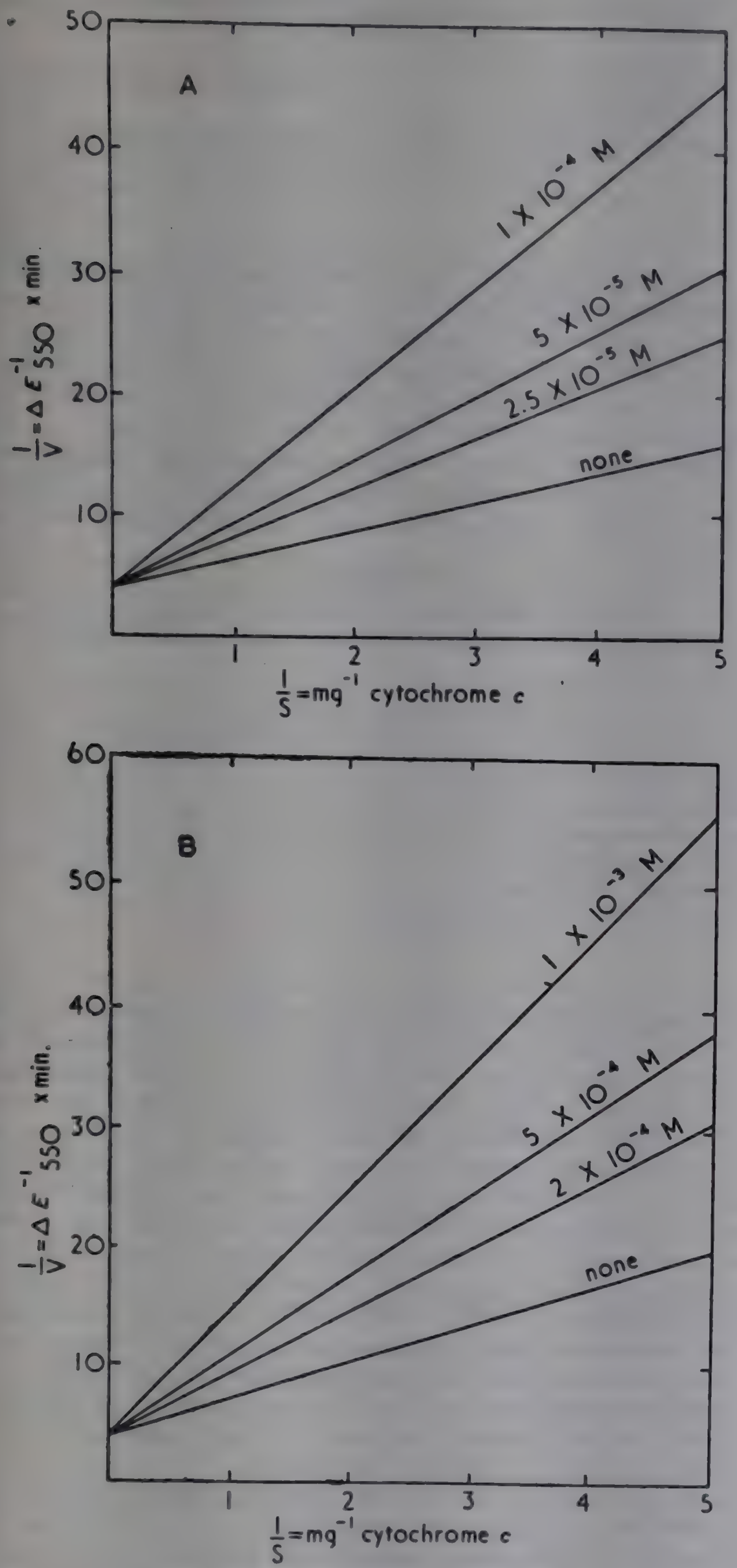



FIG. 10. — A, competitive inhibition of cytochrome *c* reduction by citrate. The standard assay system at 25° C. was employed with 1 µg. of enzyme of specific activity 200 per experiment. The cytochrome *c* concentration was varied as shown, and the different citrate concentrations are indicated on the graph. $V = \Delta E_{550}$ between 15 and 75 seconds. B, competitive inhibition of cytochrome *c* reduction by pyrophosphate. Conditions as for A. From Mahler (31).

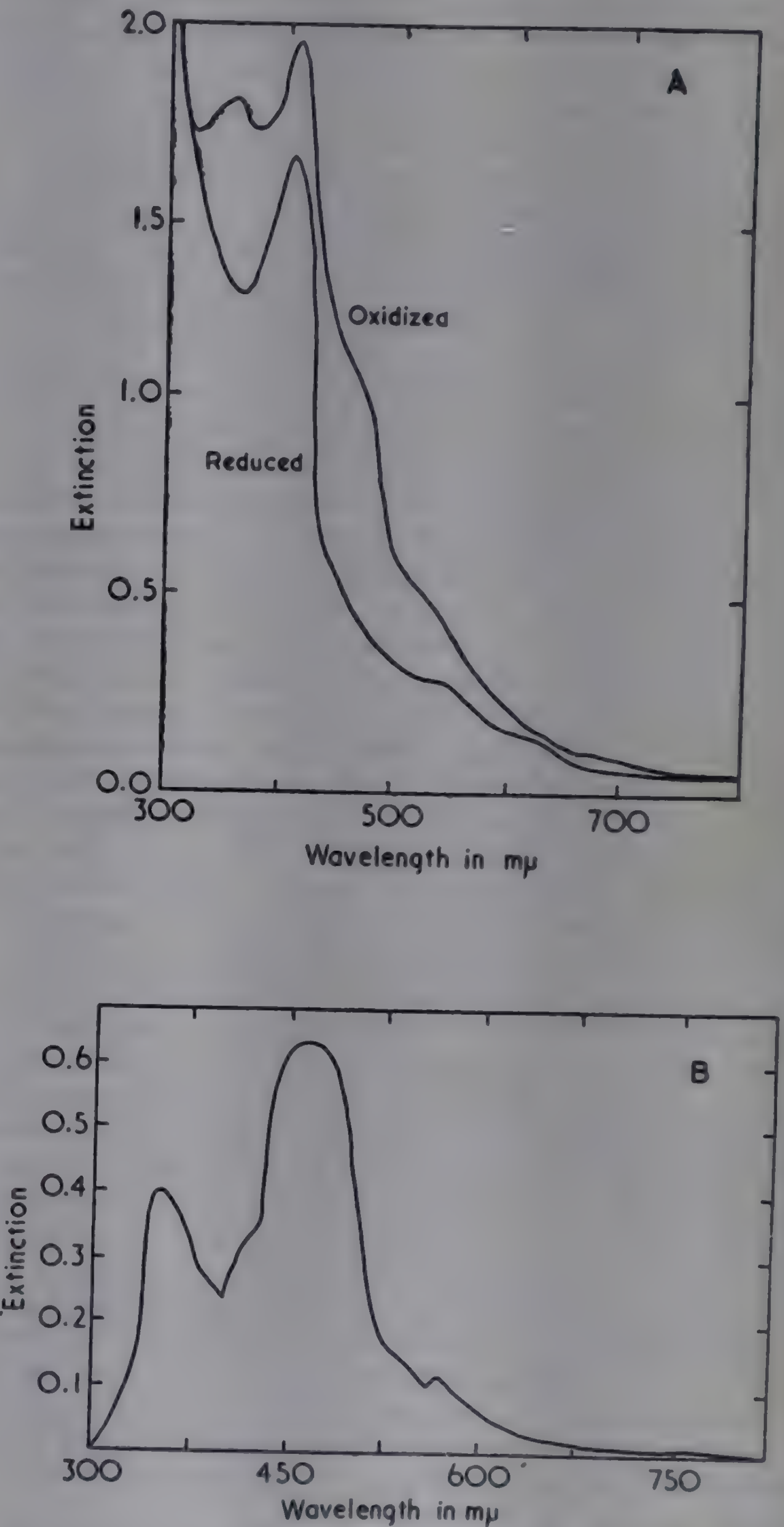



FIG. 11. — A, absorption spectrum of aldehyde oxidase. Cary spectrophotometer tracing of a 1.12 % solution of electrophoretically homogeneous enzyme. The tracing marked 'reduced' was obtained after the addition of 10 µ-moles of acetaldehyde. B, difference spectrum (oxidized minus reduced) of aldehyde oxidase. The data of A were subtracted arithmetically and replotted. From Mahler (26).

TABLE III

*Different forms of DPNH oxidase*

| Form | Name | Composition | Remarks |
|---|---|---|---|
| Flavoprotein | Diaphorase | E-FAD | Nature of flavin uncertain |
| Metalloflavoprotein | DPN-cytochrome reductase | E-FAD-$Fe_4^{+++}$ | |
| Metallo-hemoflavoprotein | | E-FAD-$Fe_{20}$-cyt.*b* | |
| Complex metallo-hemoflavo-protein | DPNH oxidase (open) | E-FAD-$Fe_{35}$-cyt.*b*-$Cu_6$-cyt.ox. | Reacts with cyt. *c* |
| ditto | DPNH oxidase (closed) | ditto | Reacts only with $O_2$ |

highly purified enzymes have been established, with each member more complex than the preceding one and each bearing a definite structural relationship to the other members in the same group. As an example of the first class we might mention liver aldehyde oxidase (26). This enzyme has the spectrum shown on the figure 11, contains FAD and molybdenum, and is capable of reducing the heme-component implicated by the spectrum, and identified as protohemin, by substrate in the presence of molybdenum. Possibly very closely related to this enzyme is the yeast lactic oxidase, recently crystallized by Appleby and Morton (27) and shown to contain FAD as well as a heme-component (previously called cytochrome $b_2$). No data concerning a possible metal component in this enzyme have as yet been forthcoming.

Two series of enzymes of increasing complexity have been studied in our laboratory. Let us center our attention on the one we have called the DPNH-oxidase series (table III). It consists of diaphorase as its simplest member, a flavoprotein without an additional component. The next step in order of complexity is the enzyme we have called DPNH-cytochrome reductase. The next step is a soluble hemo-ferro-flavoprotein isolated by Dr. Mackler (29) containing additional iron and a cytochrome *b* like pigment. Beyond this come two different forms of the enzyme both containing additional components namely cytochrome oxidase and copper, one capable of interaction with cytochrome *c*, but incapable of catalyzing the oxidation of DPNH by oxygen, while the most complex and least modified form of the enzyme is incapable of reducing cytochrome *c* but does transfer electrons exceedingly efficiently from DPNH to oxygen (29). Now I have discussed this series going from the least complex to the most complex members, while in practice we have so far only succeeded in going from the more complex to the less complex members of the group. There is good evidence that succinic dehydrogenase also may exist in forms entirely analogous to those just described, and that the old yellow enzyme and TPNH-cytochrome reductase may be the first members of yet another group. It is of considerable interest that the metal implicated as part of the metalloflavoprotein portion of the enzyme is iron in all three cases.

There is also some evidence concerning some of the other points raised earlier. Metals do form exceedingly effective chelates with riboflavin (6, 30) and even more so with RMP and FAD (31). By taking advantage of this fact we have been able to form what one may term 'artificial' metallo-, especially ferroflavoproteins which partake of some of the properties of their counterparts found in nature (31). Another line of evidence showing exceedingly tight interaction between flavoprotein and metal comes from a consideration of the redox potentials of the metalloflavoproteins (table IV). The $E_0'$ of riboflavin is —0.186 volts, while those of RMP and FAD are almost identical with that value, Association of the flavin prosthetic group with an enzyme will change this value, but it is doubtful that it might shift it more than $\pm 0.100$ volts. Indeed the value for the yellow enzyme

TABLE IV

*Oxidation potentials of flavins and flavoproteins*

| Species | $E_0'$ |
|---|---|
| Riboflavin | —0.186 |
| Flavin nucleotides (FAD, FMN) | —0.180 |
| Old yellow enzyme | —0.060 |
| Xanthine oxidase (Mo) | —0.350 |
| DPNH-cyt. reductase (Fe) | 0.00 |
| Butyryl-CoA dehydrogenase (Cu) | +0.187 |

determined by Kuhn and Boulanger is —0.060. Yet the molybdo-ferro-flavoprotein xanthine oxidase has an $E_0'$ of —0.350 volts while the $E_0'$ of the cuproflavoprotein butyryl-CoA dehydrogenase has been estimated to be +0.187 volts. This total span of some 0.530 volts gives some indication of the extent of fundamental electronic modification imposed upon flavin nucleotide by virtue of chelation with proteins and metals. Even more conclusive evidence can be adduced from a consideration of interaction functions between a series of flavoproteins with a series of structurally closely related naphthoquinones (32). Not only do metal-containing and metal-free flavoproteins show totally different patterns, but the same enzyme (*e.g.* xanthine oxidase) can be made to fall onto either one or the other of the two curves at will depending only on the presence or the absence of the metal in the medium.

The metal has also become strongly implicated in the process of oxydative phosphorylation. Several different hypotheses have been propounded (32, 33, 34, 35). A possible one is (table V) that during one part of the cyclic process catalyzed by the enzyme, namely its reduction, there takes place binding of phosphate or of phosphorylated nucleotide by the enzyme. During or just subsequent to the oxidation of the enzyme, and in the presence of an appropriate acceptor system, there will then take place either a phosphorolysis or a pyrophosphorolysis of the metal-bound anion, leading to a

TABLE V

*Effect of phosphate on metalloflavoproteins*

| Enzyme | DPNH-cytochrome reductase | Xanthine, Aldehyde oxidase, Hydrogenase |
|---|---|---|
| Metal | Fe | Mo |
| Effect of phosphate | Strong inhibitor | required |
| Stability of aquo-complex (hydroxide) of metal | ox. $K_s = 1.1 \times 10^{-36}$<br>red. $K_s = 1.6 \times 10^{-14}$ | high<br>low (?) |
| Stability of phosphate of metal | ox. low<br>red high | low (?)<br>high |
| Rate of reaction (1) | slow | fast |

(1) $Me^{+n}$ phosphate $\rightleftarrows$ aquo

$\updownarrow$

(2) $Me^{n-1}$ phosphate $\leftrightarrows$ aquo

$\Delta F^{o}$ (hydrolysis) is strongly negative

regeneration of the unphosphorylated oxidized enzyme concomitantly with transfer of phosphate to a pyrophosphorylated acceptor, *i.e.* oxidative phosphorylation.

In conclusion (figure 12) we might consider very briefly the tremendous flexibility which the presence of metal ions confer on just one system, *i.e.* the one concerned with the transfer of electrons from DPNH to the cytochrome system. One of the paths leads from the pyridine nucleotide through the flavin to the iron of the DPNH-cytochrome reductase part of the DPNH oxidase

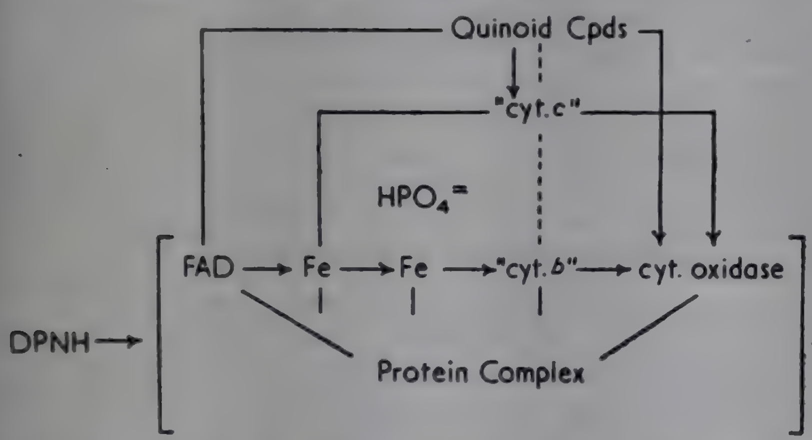



FIG. 12. — Some alternate pathways in the DPN-oxidase system.

system. From there the electrons are transfered to the 'built-in' cytochrome components, presumably through additional iron ions. This may be the process which in mitochondria leads to oxidative phosphorylation. In the presence of $CN^-$ or CO or any other agent which blocks anywhere along the chain subsequent to the metal, electrons may be diverted to external cytochrome *c* through the reductase system. If the metal in this system is blocked by a chelating agent then the electrons may be completely diverted to another parallel system, *i.e.* one involving substituted naphthoquinones, etc. If this acceptor system can in turn be made to react with one or more components of the cytochrome system then it provides an effective by path around the site of the original blocking action, a site which may originally also have been involved in oxydative phosphorylation.

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# Sur une nouvelle forme de riboflavine (vitamine $B_2$) fortement liée aux protéines (*)

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(*Reçu le 1er août 1955*)

La diversité des enzymes flaviniques est déterminée par les particularités des protéines dont elles sont composées et par les différences de structure de la partie cofermentative de molécules (mono-et dinucléotides ainsi que des nucléotides contenant les métaux).

Le caractère et la solidité de la liaison vitamine-protéine ont été beaucoup moins étudiés. Jusqu'à ces derniers temps on estimait que la liaison entre la riboflavine et la protéine se disloquait complètement par hydrolyse acide, que l'on employait comme moyen fondamental dans les méthodes chimiques courantes pour le dosage quantitatif de cette vitamine. Pour libérer la riboflavine de la forme dinucléotidique, faiblement fluorescente, on utilise en outre le traitement par des préparations de phosphatase ou par l'acide trichloracétique.

Nous avons établi que dans les tissus soumis à l'autolyse la teneur en riboflavine dosée par les méthodes existantes augmente sensiblement et que l'on obtient des résultats identiques lorsqu'on traite préalablement la matière par des préparations protéolytiques en milieu faiblement alcalin à pH 7.8 (trypsine, pancréatine, clarase, etc.) ensuite par des enzymes phosphatasiques à pH 4.5. Le tableau I illustre ce qui précède.

TABLEAU I. — *Teneur en riboflavine après divers traitements des extraits acides (en mg./g.)*

| Traitement fermentatif | | Matière | | | |
|---|---|---|---|---|---|
| Protéolyse | Phosphatase | Froment | Pois | Pomme de terre | Tissu musculaire |
| — | — | 0.66 | 1.66 | 0.51 | 0.70 |
| — | + | 1.20 | 2.33 | 0.66 | 1.57 |
| + | — | 0.90 | 1.85 | 0.66 | 1.04 |
| + | + | 3.50 | 3.70 | 2.26 | 1.98 |
| Autolyse | | 3.45 | 3.77 | 2.16 | 2.16 |

Finalement, on a mis au point une méthode permettant de doser séparément les différentes formes de riboflavine, y compris celle qui est fortement liée à la protéine.

Pour déterminer la teneur totale en riboflavine on traite l'échantillon par une solution-tampon de phosphate pH 7.8 dans un bain-marie bouillant; on le garde à l'étuve pendant 12 heures avec de la trypsine et on décompose ensuite les formes dinucléotiques par des préparations de phosphatase ou par l'acide trichloracétique, d'après Bessey, Lowry et Lowe. On dose la riboflavine dans un autre échantillon par l'une des méthodes précédemment adoptées. La différence entre les deux résultats donne la teneur en riboflavine de la forme fortement liée à la protéine.

Les essais effectués sur un grand nombre d'échantillons ont montré que la teneur totale en riboflavine déterminée par la nouvelle méthode dépassait sensiblement les teneurs en formes décomposables par les acides et dosées par les anciennes méthodes. Citons, à titre d'exemples, les quelques résultats repris au tableau II.

La forme fortement liée de la riboflavine se rencontre principalement dans les substances végétales. Sa teneur varie : elle augmente au cours de la maturation des baies, des fruits et des légumes et diminue au contraire au cours du stockage de ceux-ci. La germination des graines s'accompagne d'une néoformation active de riboflavine.

Les matières d'origine animale contiennent cette forme en quantités beaucoup plus faibles. On la trouve cependant dans les tissus musculaires, sa teneur variant d'ailleurs selon que le muscle est au repos ou travaille. Même le lait, qu'on ne supposait contenir de la riboflavine qu'à l'état libre, contient cette forme entièrement liée d'ailleurs à la fraction caséinique. La caséine, élément constitutif habituel des diètes expérimentales, s'est avérée très riche en cette forme difficile à éliminer.

Les données ci-dessus nous mènent à une conclusion ayant une grande importance pratique, qui consiste dans la nécessité de reviser entièrement le problème des sources alimentaires et fourragères de la riboflavine ainsi que les normes de sa consommation recommandées à l'homme et au bétail.

La question de savoir ce qu'est la nouvelle forme de riboflavine fortement liée aux protéines, quelle est sa nature et ses fonctions possibles se pose tout naturellement. On ne peut répondre à cette question que d'une façon préliminaire.

Nous avons isolé la forme de riboflavine fortement liée aux protéines à partir de plusieurs substances végétales par la méthode de Warburg et de Christian après en avoir éliminé toutes les formes de flavine décomposables par les acides. Les dosages spectroscopiques et chromatographiques indiquent que ces préparations contenaient du flavine-adéninedinucléotide.

Pour confirmer par ailleurs la nature dinucléotidique du pigment nous avons utilisé comme indicateur spécifique du flavine-adénine-dinucléotide un système de

(*) Le texte de cet exposé a déjà été publié, en russe et en français par les Editions de l'Académie des Sciences de l'U. R. S. S., Moscou 1955.

TABLEAU II. — *Teneur de certaines matières en diverses formes de riboflavine (en µg./g.)*

| Matières | Riboflavine libre et faiblement liée aux protéines | Riboflavine fortement liée aux protéines | Teneur totale | Rapport entre la riboflavine totale et la riboflavine labilement liée, en % |
|---|---|---|---|---|
| Forment . . . . . . . . | 1.2 | 2.3 | 3.5 | 292 |
| Maïs . . . . . . . . . . | 1.4 | 4.4 | 5.8 | 414 |
| Pommes de terre en automne . . . . . . | 0.66 | 1.6 | 2.26 | 342 |
| Pommes de terre au printemps . . . . . . | 0.64 | 0.3 | 0.94 | 147 |
| Choux . . . . . . . . . | 0.8 | 1.0 | 1.8 | 225 |
| Tomates vertes . . . . | 0.7 | 1.3 | 2.0 | 350 |
| Tomates mûres . . . . | 1.1 | 2.1 | 3.2 | 290 |
| Pommes . . . . . . . | 0.7 | 0.67 | 1.37 | 196 |
| Tissu musculaire . . . . | 1.57 | 0.41 | 1.98 | 125 |
| Foie . . . . . . . . . | 40.0 | 6.6 | 46.6 | 116 |
| Levure . . . . . . . . . | 9.95 | 0.85 | 10.8 | 108 |
| Lait . . . . . . . . . | 1.24 | 0.18 | 1.42 | 114 |
| Caséine . . . . . . . . | 4.3 | 5.7 | 10 | 232 |

D-amino-acide-oxydases. L'enzyme et l'apoenzyme ont été obtenus par la méthode de Krebs. On a pu réactiver complètement l'apoenzyme par l'addition de la préparation isolée, alors que le mononucléotide de la riboflavine isolée de la même matière (feuilles) n'a pas cette activité. Ainsi la nouvelle forme est bien un flavine-adénine-dicléotide.

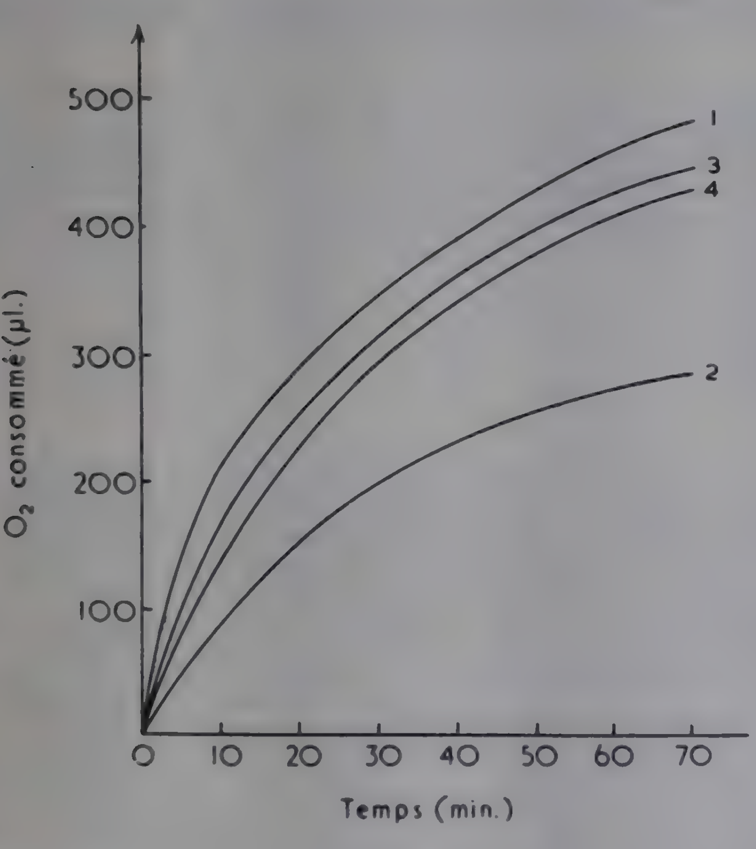


*Fig.* 1. — Activité de la succino déshydrogénase : (1) témoins, (2) après irradiation de 15 min. par les rayons ultra-violets, (3) réactivation par addition de dinucléotide de flavine et d'adénine, extrait de la levure, (4) réactivation par addition de préparations extraites des feuilles, contenant ce dinucléotide.

Il reste cependant à savoir, si le coenzyme natif contient un métal qui aurait pu être libéré au cours du traitement. On devra considérer comme possible une telle éventualité si l'on tient compte des derniers travaux de Mackler, Mahler, Bock, Green et autres, sur la détection et les propriétés des flavoprotéines contenant des métaux.

Comme le coenzyme que nous avons découvert est très fortement lié aux protéines, il serait naturel de se demander s'il ne constitue pas un groupement prosthétique d'enzymes fortement liés aux structures cellulaires et qui ne peuvent pas passer en solution. Parmi ces enzymes nous avons surtout porté notre attention sur la succinodéshydrogénase, dont la nature flavoprotéique reste encore discutable.

Les préparations de succinodéshydrogénase ont été obtenues à partir du muscle cardiaque du veau et du muscle pectoral du pigeon. Dans le dernier cas, les préparations sont beaucoup plus actives, une fois et demie ou deux fois, si les pigeons étaient préalablement soumis à une diète à forte teneur (et non pas faible) en riboflavine. On connaissait déjà cette indication indirecte de la nature flavinique de la succinodéshydrogénase. Il importe seulement de noter dans ce cas que la teneur en forme fortement liée de la riboflavine est aussi plus élevée chez les pigeons soumis à une diète riche en riboflavine (1.7 µg. au lieu de 0.6 µg.).

Les préparations de succinodéshydrogénase obtenues d'après Stotz et Hastindos pour l'élimination des formes labilement liées de la riboflavine ont été soumises à la dialyse, ensuite à la macération dans l'acide acétique faible (pH 4) et à une seconde dialyse. La forme fortement liée de la riboflavine demeure dans ce cas entièrement dans le précipité formé après la dialyse et son activité fermentative accroît au cours de la purification.

On dépose le précipité purifié dans des boîtes de Petri

avec une solution-tampon phosphate pH 7.4 en une couche de 4 mm. d'épaisseur et on l'irradie pendant des périodes variables avec des rayons ultra-violets. L'irradiation pendant 45 min. rend l'enzyme absolument inactif et l'addition de préparations dinucléotiques de flavine extraites des feuilles n'exerce sur lui aucune action. Une irradiation de 5 min. n'inactive pas l'enzyme. Un traitement de 15 min. par des rayons ultra-violets donne une inactivation marquée, et une addition des préparations indiquées ci-dessus rétablit presque complètement l'activité de l'enzyme ainsi que le montre la figure 1.

Les résultats expérimentaux exposés plaident, avant tout, en faveur de la nature flavinique de la succino-déshydrogénase. Ils prouvent en outre que la forme adéninedinucléotidique de la riboflavine fortement liée aux protéines et que nous avons découverte constitue un coenzyme de ce système. Les recherches ultérieures nous montreront si son rôle se limite à cette fonction ou s'il est plus étendu.

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# О НОВОЙ ПРОЧНО СВЯЗАННОЙ С БЕЛКОМ ФОРМЕ РИБОФЛАВИНА (ВИТАМИНА $B_2$)

по В. Н. БУКИН

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Разнообразие флавиновых ферментов обусловливается как специфическими особенностями белков, входящих в их состав, так и различием в строении коферментной части молекул (моно-, ди- и металлсодержащие нуклеотиды).

Значительно менее изучен вопрос о характере и прочности связи витамин — белок. До последнего времени считалось, что связь рибофлавина с белком полностью расщепляется при кислотном гидролизе, который и применяется в качестве основного приема в существующих химических методах количественного определения этого витамина. С целью освобождения рибофлавина из динуклеотидной, слабо флюоресцирующей формы применяется, кроме того, обработка фосфатазными препаратами или трихлоруксусной кислотой.

В нашей лаборатории было установлено (К. Л. Поволоцкая), что в тканях, подвергаемых автолизу, количество определяемого по существующим методам рибофлавина значительно возрастает, и такие же результаты получаются, если материал сначала подвергают обработке протеолитическими препаратами в слабо щелочных условиях при pH 7.8 (трипсин, панкреатин, клараза и др.), а затем фосфатазными ферментами при pH 4.5. Сказанное поясняет табл. 1.

Таблица 1

*Количество определяемого рибофлавина при различной обработке кислотных экстрактов, µг/г*

| Ферментативная обработка | | Объект | | | |
|---|---|---|---|---|---|
| протеолиз | фосфатаза | пшеница | горох | картофель | мышца |
| — | — | 0.66 | 1.66 | 0.51 | 0.70 |
| — | рт | 1.20 | 2.33 | 0.66 | 1.57 |
| рт | — | 0.90 | 1.85 | 0.66 | 1.04 |
| рт | рт | 3.50 | 3.70 | 2.26 | 1,98 |
| Автолиз | | 3.45 | 3.77 | 2.14 | 2.16 |

В итоге был разработан метод, позволяющий проводить раздельное определение различных форм рибофлавина, в том числе и прочно связанной с белком формы. Для определения общего содержания рибофлавина материал экстрагируют фосфатным буфером при pH 7.8 на кипящей водяной бане, инкубируют 12 часов с трипсином, а затем динуклеотидные формы разлагают фосфатазными препаратами или трихлоруксусной кислотой по Бессей, Лоури и Лоув (Bessey, Lowry a. Love). В параллельной пробе рибофлавин определяется по одному из ранее принятых методов. Разница между определениями дает содержание прочно связанной с белком формы.

Проведенное испытание большого числа объектов показало, что общее содержание рибофлавина, определяемое по новому методу, существенно превосходит те количества кислотноотщепляемых форм, которые определялись прежними методами. Для иллюстрации в табл. 2 приведены некоторые примеры.

Таблица

*Содержание различных форм рибофлавина в некоторых объектах, µг/г*

| Объект | Рибофлавин | | | |
|---|---|---|---|---|
| | свободный и непрочно связанный с белком | прочно связанный с белком | общее содержание | отношение общего к непрочно связанному, % |
| Пшеница | 1.2 | 2.3 | 3.5 | 292 |
| Кукуруза | 1.4 | 4.4 | 5.8 | 414 |
| Картофель осенью | 0.66 | 1.6 | 2.26 | 342 |
| » весной | 0.64 | 0.3 | 0.94 | 147 |
| Капуста | 0.8 | 1.0 | 1.8 | 225 |
| Томаты незрелые | 0.7 | 1.3 | 2.0 | 350 |
| зрелые | 1.1 | 2.1 | 3.2 | 290 |
| Яблоки | 0.7 | 0.67 | 1.37 | 196 |
| Мышца | 1.57 | 0.41 | 1.98 | 125 |
| Печень | 40.0 | 6.6 | 46.6 | 116 |
| Дрожжи | 9.95 | 0.85 | 10.8 | 108 |
| Молоко | 1.24 | 0.18 | 1.42 | 114 |
| Казеин | 4.3 | 5.7 | 10' | 232 |

Прочно связанная форма рибофлавина встречается преимущественно в растительных объектах, причем количество ее изменяется — увеличивается при созревании ягод, плодов и овощей и снижается при их хранении; при прорастании семян идет энергичное ее новообразование.

Животные объекты содержат гораздо меньше этой формы рибофлавина, но все же она обнаруживается в мышцах и притом в различном количестве в зависимости от того, находится ли мышца в покое или выполняет работу. Даже молоко содержит эту форму рибофлавина, причем она целиком связана с казеиновой фракцией, хотя ранее считалось, что рибофлавин присутствует в молоке лишь в свободном состоянии. Казеин — обычная составная часть экспериментальных диет — оказался весьма богат этой трудно удаляемой формой.

Из приведенных данных напрашивается вывод, имеющий большое практическое значение. Он сводится к необходимости заново пересмотреть вопрос о пищевых и кормовых источниках рибофлавина и тех нормах его потребления, которые рекомендованы для человека и сельскохозяйственных животных.

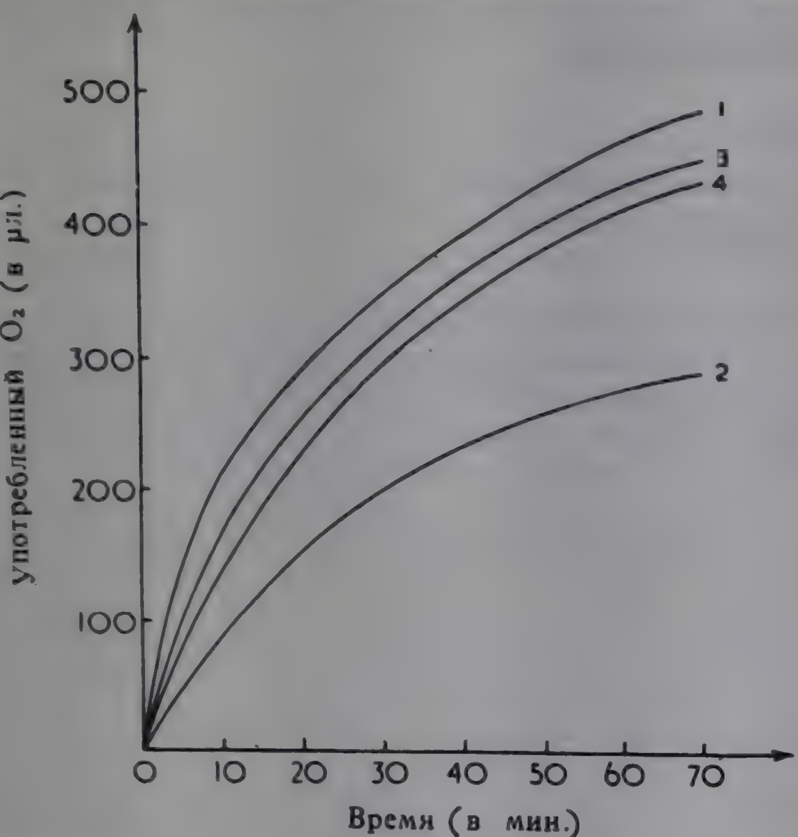
Естественно возникает также вопрос, что же представляет

собой новая, прочно связанная с белком форма рибофлавина, какова ее природа и возможные функции. На этот вопрос можно дать ответ лишь в предварительном порядке.

Прочно связанная с белком форма рибофлавина была выделена нами из ряда растительных объектов по методу Варбурга и Христиана после предварительного удаления из них всех кислотноотщепляемых форм флавина.

Спектроскопические и хроматографические определения указывали на наличие в препаратах флавинадениндинуклеотида.

Для дальнейшего подтверждения динуклеотидной природы




Сукциндегидразная активность: (1) начальная, (2) после 15 мин. облучения УФ, (3) после добавления флавинадениндинуклеотида, выделенного из дрожжей, (4) после добавления динуклеотидного препарата флавина, выделенного из листьев

пигмента использовалась система d-аминооксидаз как специфический индикатор на флавинадениндинуклеотид. Фермент и апофермент были получены по методике Кребса. Апофермент удалось полностью реактивировать добавлением выделенного препарата, в то время как выделенный из того же объекта (листьев) мононуклеотид рибофлавина не обладал этой активностью. Таким образом, новая форма представляет собой флавинадениндинуклеотид.

Остается, однако, открытым вопрос, не содержится ли в нативном коферменте тот или иной металл, который мог отщепиться в процессе обработки. Такую возможность следует иметь в виду при дальнейших исследованиях, учитывая последние работы Маклера, Малера, Бока, Грина и др. (Mackler, Mahler, Bock, Green a. al.) по обнаружению и свойствам металлсодержащих флавопротеинов.

Так как обнаруженный нами кофермент весьма прочно связан с белком, естественно было предположить, не является ли он простетической группой тех ферментов, которые прочно связаны с клеточными структурами и не могут быть переведены в раствор. Среди таких ферментов наше внимание было сосредоточено на сукциндегидразе, флавопротеиновая природа которой до сих пор остается спорной.

Препараты сукциндегидразы получали из сердечной мышцы теленка и грудной мышцы голубя. В последнем случае препараты были значительно активнее (в 1.5—2 раза), если голуби предварительно находились на диете с высоким содержанием рибофлавина, а не с низким. Подобное косвенное указание о флавиновой природе сукциндегидразы имелось и ранее, в данном случае важно лишь отметить, что содержание прочно связанной формы рибофлавина также было выше у голубей, содержавшихся на диете, богатой рибофлавином (1.7 $\mu$г вместо 0.6 $\mu$г-г).

Полученные по Штоц и Хастингсу (Stotz a. Hastings) препараты сукциндегидразы с целью удаления непрочно связанной формы рибофлавина были подвергнуты диализу, настаиванию со слабой уксусной кислотой (pH 4) и вторичному диализу. Прочно связанная форма рибофлавина при этом целиком оставалась в выпавшем после диализа осадке, а его ферментативная активность по мере очистки возрастала.

Очищенный осадок помещали в чашки Петри с фосфатным буфером pH 7.4 слоем в 4 мм и освещали ультрафиолетовым светом в течение различного времени. Облучение в течение 45 минут полностью инактивировало фермент, и добавление к нему выделенных из листьев динуклеотидных препаратов флавина не оказывало никакого действия. При 5-минутном освещении инактивации не наблюдалось. Освещение в течение 15 минут давало заметное инактивирование, при котором добавка указанных препаратов почти полностью реактивировала фермент, как это видно из рис. 1.

Изложенные результаты опытов прежде всего говорят в пользу флавиновой природы сукциндегидразы, а также о том, что коферментом этой системы является обнаруженная нами прочно связанная с белком адениндинуклеотидная форма рибофлавина. Ограничивается ли ее роль лишь этой функцией или она шире — задача дальнейших исследований.

# Respiratory chain phosphorylation

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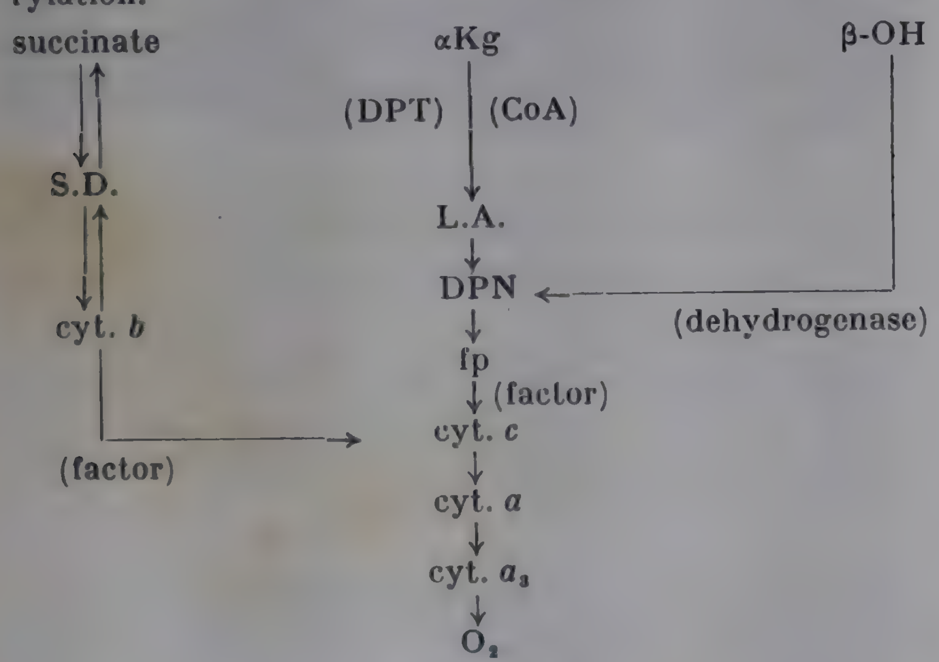
(*Received* 9 *April* 1955)

## INTRODUCTION

Respiratory chain phosphorylation was discovered by Belitzer and Tsibakowa (1) and Ochoa (2, 3), when they found that more than one atom of phosphorus is esterified for each atom of oxygen consumed by respiring tissue preparations. It was first demonstrated directly by Friedkin and Lehninger (4) using reduced diphosphopyridine nucleotide (DPNH) as hydrogen donor. It is now known that the greater part of oxidative phosphorylation in aerobic cells occurs in the respiratory chain.

### *Respiratory chain*

General agreement has not yet been reached on the constitution of the respiratory chain, either in non-phosphorylating mitochondrial fragments, or in the intact mitochondria. This is not the place to discuss the different views in detail, and we shall use scheme A as the basis for discussing respiratory chain phosphorylation.




Scheme A

($\alpha$Kg = $\alpha$-ketoglutarate ; CoA = coenzyme A ; DPN = diphosphopyridine nucleotide ; fp = flavoprotein ; cyt. = cytochrome ; S. D. = succinic dehydrogenase ; L. A. = $\alpha$-lipoic acid ; $\beta$-OH = $\beta$-hydroxybutyrate ; DPT = diphosphothiamine)

Some explanations are required :

— $\beta$-OH is given only as an example of a number of substrates which react through DPN in this way. A number of other substrates react through triphosphopyridine nucleotide by a similar pathway.

— Components of the chain are defined as substances which undergo alternate reduction and oxidation as the hydrogen atoms of the substrate (or their electrons) pass to oxygen. Other substances which have been identified as necessary for these reactions, but which do not themselves undergo oxido-reduction, *e.g.* CoA and DPT in the reaction between $\alpha$Kg and L. A., are placed in brackets over the arrow denoting the hydrogen or electron transfer. (The enzymes involved in the reaction between $\alpha$Kg and L. A. have been omitted owing to lack of space). Similarly $\beta$-OH dehydrogenase has been placed in brackets, since it has not been established whether or not this undergoes an oxido-reduction.

— There is quite good evidence from a number of workers that in mitochondria or mitochondrial fragments, both cytochrome *b* and flavoprotein require an intermediate unidentified factor for their reaction with cytochrome *c* (5, 6, 7, 8, 9). In earlier papers (6, 7), it was assumed that this factor operated as an additional electron-transferring step between flavoprotein or cytochrome *b* and cytochrome *c*. While this may be true, there is no direct evidence that it acts in this way ; its rôle may be more analagous to that of coenzyme A.

— The reactions between $\alpha$Kg and DPN have been established by the work of Gunsalus (10) with extracts prepared from micro-organisms, and there is good reason to believe that the same mechanism applies to animal cells. The pathway between DPN and succinate and oxygen has been elucidated by experiments with non-phosphorylating heart sarcosomal (mitochondrial) fragments (11, 6, 7, 12). Chance (13) has suggested that cytochrome *b* is not on the main pathway for the oxidation of succinate by sarcosomal fragments. If this is the case, this portion of the chain should be rewritten as in scheme B :

$$\begin{array}{c} \text{Succinate} \rightleftarrows \text{S.D.} \xrightarrow{\text{(factor)}} \text{cyt. } c \\ \uparrow\downarrow \\ \text{cyt. } b \end{array}$$

**Scheme B**

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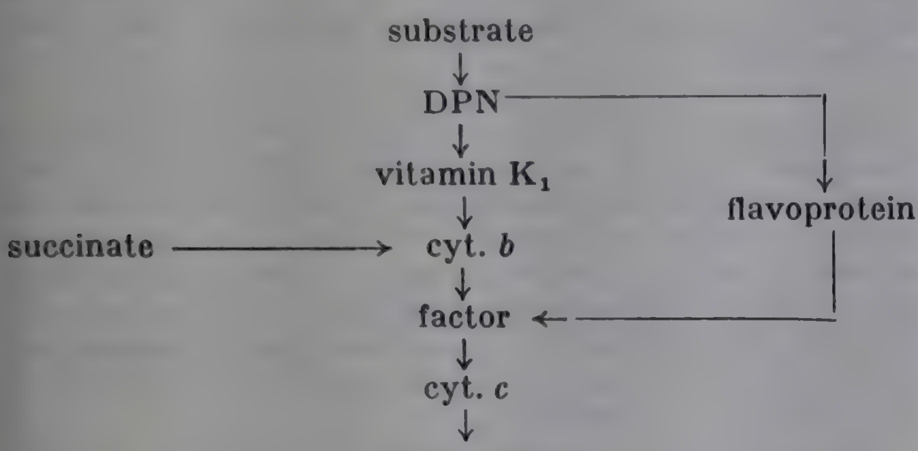
— The question arises whether the respiratory chain found in the sarcosomal fragments is the same as that in the intact sarcosome or mitochondria. Chance and Williams (14) consider that, although cytochrome *b* is not involved in the oxidation of DPNH (7) or of succinate (13) by sarcosomal fragments, it is involved in these reactions in the intact mitochondria. They write the respiratory chain for β-hydroxybutyrate, thus :

$$\beta\text{-OH} \rightarrow \text{DPN} \rightarrow \text{fp} \rightarrow \text{cyt.}\ b \rightarrow \text{cyt.}\ c \rightarrow \text{cyt.}\ a \rightarrow \text{cyt.}\ a_3 \rightarrow O_2$$

Scheme C

An alternative explanation of their findings is that the same direct pathways occur in both the intact mitochondria and in the non-phosphorylating fragments, but that the side-paths are much more rapid in the intact mitochondria.

Martius (15) also believes that cytochrome *b* is involved in the oxidation of DPN by the mitochondria. In his scheme (D), vitamin $K_1$ and not flavoprotein is the immediate acceptor of the hydrogen atoms of DPNH, in the phosphorylating system. The pathway through flavoprotein is non-phosphorylative.




Scheme D

*Number of phosphorylative steps*

The problem with which this paper is mainly concerned is the location of those steps in the respiratory chain which are associated with phosphorylation. First, it would be helpful to know how many steps are phosphorylative. A useful guide for this purpose is given by measurements of the yield of oxidative phosphorylation obtained in the oxidation of different substrates. Since (according to scheme A) different substrates, *e.g.* α-ketoglutarate, β-hydroxybutyrate and succinate, enter the respiratory chain at different points, the different yields might be expected to tell us something about which steps are phosphorylative. This is one of the reasons why so much effort has been put into determining the actual yields which are expressed as P : O ratios (number of atoms of phosphorus esterified per atom of oxygen consumed).

It is likely that only one atom of phosphorus is esterified in each step. Although the reaction :

$$\text{AMP} + \text{pyrophosphate} \rightarrow \text{ATP}$$

(AMP = adenosine monophosphate ; ATP = adenosine triphosphate)

could cause the incorporation of two atoms of phosphorus, this type of reaction does not appear to proceed in oxidative phosphorylation. This is shown by Lehninger and Smith's demonstration that inorganic pyrophosphate was not an intermediate (16), and the fact that, in the absence of myokinase, AMP is phosphorylated very slowly in comparison with ADP (figure 1 ; see (17) for a review of previous work on this subject).

It is also clear from figure 1 that the synthesis of ATP commences immediately the reactants are brought together without any time lag (the concentration of sarcosomal protein was only 0.12 mg./ml. in this experiment). This shows that intermediate phosphate compounds do not accumulate in any appreciable amounts.

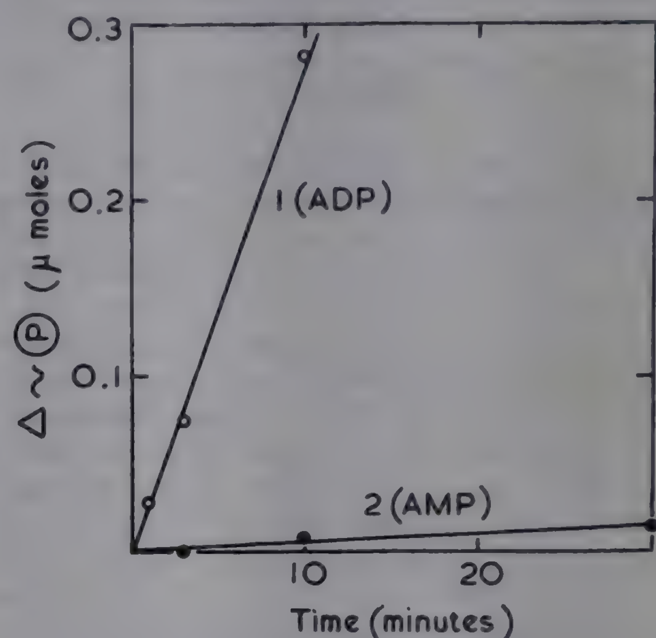



FIG. 1. — Rate of phosphorylation of ADP (curve 1) and AMP (curve 2) by cat-heart sarcosomes oxidizing α-ketoglutarate. Sarcosomes isolated in 0.28 M sucrose, 0.01 M ethylenediaminetetraacetate (EDTA), pH 7.4. Reaction mixture : phosphate, 0.03 M ; malonate, 0.01 M ; fluoride, 0.04 M ; glucose, 0.018 M ; EDTA, 0.001 M ; AMP, $6 \times 10^{-4}$ M ; ADP, $6 \times 10^{-4}$ M ; magnesium, 0.005 M ; sarcosomes, 0.12 mg. protein/ml. ; α-ketoglutarate, 0.005 M ; cytochrome *c*, $4.8 \times 10^{-5}$ M. From Slater and Holton (17).

Measurements of P : O ratios have another object in view. It is important for our knowledge of cell physiology to determine how much ATP is synthesized by intracellular respiration, and the only method at present available is to measure the P : O ratios with isolated mitochondria. It is, of course, possible that the isolation procedure might lower the P : O ratio, and as mild conditions as possible should be used and the effect of varying the isolation procedure should be studied.

*α-Ketoglutarate.* — Some recent measurements of the P : O ratio with α-ketoglutarate as substrate are summarized in table I. Mitochondria rapidly oxidize the succinate formed from the oxidation of α-ketoglutarate, but this reaction can be greatly slowed by the addition of malonate which has no effect on the phosphorylation. In our hands, malonate is not completely effective in stopping the oxidation at the succinate stage in heart sarcosomes (it is even less effective in the case of blowfly sarcosomes, 23). Since the oxidation of succinate to

TABLE I.

*Mean values of the phosphorylation ratios for the oxidation of α-ketoglutarate by rat liver mitochondria and heart sarcosomes, in the presence of fluoride and malonate*

| Tissue | Reference | Temp. (° C.) | $QO_2$ | O : αKg | P : O | P : αKg |
|---|---|---|---|---|---|---|
| Liver | Copenhaver and Lardy (18) | 30 | 10-15 | 0.93 | 3.6 | 3.3 |
| Heart | Maley and Plaut (19) | 30 | 33-67 | 1.00 | 3.33 | 3.33 |
| | Maley and Plaut (20) | 15-30 | — | — | 3.32 | — |
| | Slater and Holton (21) (*) | 25 | 83 | 1.14 | 2.73 | 3.07 |
| | Slater (22) (**) | 25 | 91 | 1.13 | 2.86 | 3.22 |

(*) The figures given here are the means of values obtained for preparations isolated in 0.28-0.32 M sucrose, 0.005-0.01 M ethylenediaminetetraacetate (EDTA), pH 7.4, which are included in this paper (one abnormally low value omitted from mean). 14 measurements of P : O ratio, including 9 measurements of O : αKg and P : αKg ratios.

(**) A reinvestigation, using 0.21 M sucrose, 0.01 M EDTA, pH 7.4, as isolation medium. 19 measurements of P : O ratio, including 9 measurements of O : αKg and P : αKg ratios. The P : O ratios varied between 2.74 and 2.97, the P : αKg between 2.99 and 3.49.

fumarate has a low P : O ratio, the measured P : O ratio slightly underestimates the ratio for the oxidation of α-ketoglutarate to succinate, and the P : αKg ratio overestimates it. The true ratio will lie somewhere between the two values.

For heart, there is quite a small discrepancy between the figures of Maley and Plaut and ours, of little importance from the point of view of the assessment of the yield of phosphorylation *in vivo*. But the difference is important from the point of view of the present discussion. Our results suggest that the ratio is very close to 3, which could mean that only 3 steps in the chain between α-ketoglutarate and oxygen are phosphorylative. However, the results are also consistent with the possibility that there are 4 steps, and there are side-reactions so that each phosphorylative step gives an average P : O ratio of 0.75. The type of side-reaction envisaged is the hydrolysis of an intermediate in the phosphorylation reaction instead of reaction with inorganic phosphate or ADP, for example the hydrolysis of succinyl-coenzyme A. The hydrolysis of ATP is not included among the possible side-reactions, since it is subsequent to the oxidative phosphorylation reactions and it is merely a technical problem to ensure that the ATP formed is measured before it is hydrolysed. This can be achieved by adding sufficient hexokinase (17, 23), or by isotopic methods (24).

If the measured P : O ratio is above 3 by an amount exceeding the experimental error, it proves that at least 4 steps in the chain are phosphorylative. Maley and Plaut's value for the P : O ratio is about 10 % above 3, and Copenhaver and Lardy's value for liver is about 20 % above 3. The disagreement between our results and those of the Wisconsin workers is very small when the P : αKg ratios are considered. This suggested to us that the discrepancy lay in the measurement of oxygen uptake and, in our hands, Copenhaver and Lardy's (18) procedure under-estimated the oxygen uptakes, thereby over-estimating the P : O ratios. Copenhaver and Lardy (25) have replied to our criticism of their procedure, and further discussion of this question is outside the scope of this paper. Perhaps, further consideration should be given to the possibility that there is a real difference between liver and heart.

β-*Hydroxybutyrate.* — Copenhaver and Lardy (18) find a P : O ratio of 2.5 in liver mitochondria with this substrate, and Maley and Plaut (19) have reported similar values for heart. These results suggest that there are at least three steps in the chain between β-hydroxybutyrate and oxygen associated with phosphorylation.

*Succinate.* — Succinate is a particularly interesting substrate from the point of view of the P : O ratio, since, according to scheme A, its oxidation does not involve pyridine nucleotide, and there is no convincing evidence that it involves flavine. Unfortunately, there is no satisfactory inhibitor of the oxidation of the fumarate formed from the succinate, and a considerable proportion of the oxygen uptake may be associated with the oxidation of fumarate.

Phosphorylation coupled with the oxidation of succinate was first shown by Belitzer and Tsibakowa (1) using inhibition by arsenite to prevent oxidation of malate (see also 26). Green (27) obtained phosphorylation (P : O = 0.38) with partially disrupted mitochondria, which did not oxidize fumarate. There is, therefore, no doubt that phosphorylation is associated with the oxidation of succinate to fumarate, but the P :O ratio is uncertain. Copenhaver and Lardy (18) obtained a mean P : O ratio of 1.7 with liver mitochondria oxidizing succinate, and Maley and Plaut's (20) value for heart was 1.64, but in neither case is it possible to calculate the ratio for the oxidation of succinate to fumarate. These values must be regarded as upper limits, and cannot be taken to demonstrate that more than one step in the succinic oxidase system is phosphorylative. The P : O ratio found by Krebs *et al.* (24) with liver homogenates is higher, but since their method measures the rate of incorporation of $^{32}P$ into ATP, these values cannot be used to determine the number of phosphorylative steps. There is, in fact, a possibility that a considerable proportion of the incorporation of $^{32}P$ into ATP in this experiment is due to the reaction, discovered by Kaufman (28), between ATP, succinate and coenzyme A, and is not connected with the oxidation reaction (29, 21, 30).

Our sarcosomal preparations give a P : O ratio of about 1 with succinate (31). However, it will be shown below that there is some evidence that there are two phosphorylative steps in this reaction.

### *Location of phosphorylative steps*

Belitzer and Tsibakowa (1), who first clearly showed that phosphorylation occurred in the respiratory chain, also made the first experimental attempt to locate the phosphorylative steps in the chain. They found no phosphorylation with *p*-phenylenediamine as substrate and concluded that the 'iron system' was not coupled with phosphorylation. Ochoa (32) was unable to find any phosphorylation with DPNH as substrate.

*Thermodynamic considerations.* — Lipmann (33) discussed the location of the phosphorylation steps from the point of view of the thermodynamics of the respiratory chain. The synthesis of energy-rich phosphate bonds, averaging about 12 000 calories per bond, is equivalent to a span of oxidation-reduction potential of about 0.25 volt for a two-electron system. The available potential between oxygen and a pair of average substrate hydrogens is about 1.2 volt. On the basis of Ochoa's experimental P : O ratio of 3, Lipmann cut out from the potential gradient three 0.25 volt portions in succession, and concluded that at potential levels of around —0.3, +0.1 and +0.5 volt with reference to the normal hydrogen electrode, the pair of electrons is intercepted three times in succession by chemical devices which transform 0.25 volt portions into energy-rich phosphate bonds. According to Lipmann's scheme, these three potential levels correspond to the pyridine nucleotides, flavoprotein and cytochrome respectively.

The thermodynamic approach to this question has some severe limitations, which were clearly expressed by Ogston and Smithies (34). Moreover, the nature of the respiratory chain imposes what might be called « stoicheiometric » limitations on the location of the phosphorylation steps, which have to be considered as well as the thermodynamic aspects. Two electron steps do not occur all along the respiratory chain. When the chain reaches the cytochromes, there is a single electron transfer, and a difference of oxidation-reduction potential of 0.5 volt between two cytochromes is required to yield an energy-rich phosphate bond, according to Lipmann's formulation. Moreover, if the direct reaction between two cytochromes is associated with phosphorylation, this reaction would occur twice for each pair of hydrogen atoms or electrons and would account for two of the phosphorylative steps in the respiratory chain. In the reaction with oxygen, the conventional practice of thinking of P : O ratios is somewhat misleading, because the reaction is with the oxygen molecule, not the atom.

$$4a_3^{++} + 4H^+ + O_2 \rightarrow 4a_3^{+++} + 2H_2O$$

Since only one oxygen molecule is involved on the left-hand side of this equation, one atom of phosphorus would satisfy the requirements of stoicheiometry. This would give a P : O ratio of 1 or a P : O ratio of 0.5. If this were the case, the P : O ratio obtained would not be a whole number, quite apart from the question of side-reactions.

### *DPNH → $O_2$*

The first success in a direct experimental approach to this problem was obtained in 1949 by Friedkin and Lehninger (4), who demonstrated phosphorylation coupled with the oxidation of DPNH by liver mitochondria. Later work of Lehninger (35) showed that it was necessary to swell the mitochondria by a preliminary hypotonic treatment in order to demonstrate this phosphorylation. This might be the explanation of the zero phosphorylation obtained by Ochoa with DPNH (32), since the dialysed heart extracts he used probably contained normal sarcosomes.

Lehninger (35) compared the phosphorylation with DPNH and with β-hydroxybutyrate as substrates, using these swollen mitochondria (see table II). He concluded

TABLE II.
*Comparison of phosphorylation ratios with β-hydroxybutyrate and DPNH as substrates*

| Expt. | DPNH | | β-hydroxybutyrate | |
|---|---|---|---|---|
| | Time (min.) | P : DPNH | Time (min.) | P : AcAc |
| 1 | 7 | 1.32 | 30 | 1.50 |
| | 11 | 1.18 | | |
| 2 | 10 | 1.48 | 32 | 1.54 |
| | 14 | 1.40 | | |

Lehninger's experiments with hypotonically-treated liver mitochondria (35).

that the phosphorylation ratio found with DPNH was near enough to that obtained with β-hydroxybutyrate to warrant the conclusion that all the phosphorylation steps associated with the oxidation of β-hydroxybutyrate occurred in the reaction between DPNH and oxygen, and none between substrate and DPN. This was confirmed by the finding (36) that there was no phosphorylation associated with the reaction scheme :

$$\beta\text{-OH} \rightarrow \text{DPN} \rightarrow \text{oxaloacetate}$$

It follows, then, that if there are three phosphorylative steps in the reaction between β-hydroxybutyrate and oxygen (see above), there must be three steps between DPNH and oxygen. But this has not yet been demonstrated directly ; Lehninger's (35) highest ratio was 1.89.

### *α-Kg → DPN (substrate-linked phosphorylation)*

In 1949, Hunter (37) found phosphorylation (P : αKg = 0.8 — 0.9) associated with the reaction scheme :

$$\alpha\text{Kg} \rightarrow \text{DPN} \rightarrow \text{oxaloacetate}$$

Since Lehninger (36) had excluded phosphorylation in the reaction DPNH → oxaloacetate, it followed that, in the α-ketoglutaric oxidase system, some phosphorylation

must occur between substrate and DPN. Kaufman *et al.* (28, 38) and Sanadi (39) found that the esterification of phosphate occurred by reaction between energy-rich succinyl-CoA, ADP and inorganic phosphate thus :

$$\text{Succinyl-CoA} + \text{ADP} + H_3PO_4 \rightleftarrows \text{Succinate} + \text{ATP} + \text{CoA}$$

Guanosine diphosphate has been identified as an intermediate of this reaction (40).

*Substrate → cyt. c*

In 1950, phosphorylation was demonstrated with ferricytochrome *c* as hydrogen acceptor, using α-ketoglutarate as substrate and heart-muscle sarcosomes as the enzyme system (41). The primary phosphate acceptor was ADP, and the ATP formed transferred its terminal phosphate to glucose by means of yeast hexokinase added to the reaction mixture. The hexosemonophosphate (HMP) formed was estimated by a sensitive spectrophotometric method (42, 43) based on Racker's(44) method of estimating phosphohexokinase activity. The P : O ratio (number of atoms of phosphorus esterified per oxygen atom equivalent of the ferricytochrome *c*) was 2.16, and there was reason for believing that this value might be an underestimate. Since the P : O ratio with oxygen as hydrogen acceptor under the same conditions was 2.5, it appeared reasonable to conclude that all the phosphorylation steps occurred between substrate and ferricytochrome *c*. Additional experiments under more strictly controlled conditions have been recently published (45). Although these results do not differ very much from the earlier figures, the divergence is sufficient to make the original conclusion inadmissable. In the first place, the P : O ratio for the aerobic reaction with our heart sarcosomes is about 3 (table I), rather than 2.5 (no malonate was used in the 1950 experiments). Secondly, the P : O ratios for the anaerobic reaction between α-ketoglutarate and ferricytochrome *c* are a little lower than the 2.16 previously found. In 9 experiments, the mean P : O ratio was 1.72 (range, 1.5-1.9) and the mean P : αKg ratio 1.80 (range 1.6-2.0).

In a typical experiment, a spectrophotometric Thunberg tube, consisting of a Thunberg tube sealed on to a 1 cm. silica cell, and fitting into the cell-holder of a spectrophotometer, was filled with the reaction mixture described in table III. A sarcosomal suspension (0.2 ml. containing 1.4 mg. protein) was placed in the hollow stopper which was kept in ice during the evacuation of the Thunberg tube. Special care is necessary to remove all the oxygen. At zero time, the sarcosomal suspension was mixed with the reaction mixture, and the reduction of cytochrome *c* followed by measuring the optical density at 580 mμ against a reference cell containing all the components of the reaction mixture, except the cytochrome *c*. When the decrease of the optical density due to the reduction of cytochrome *c* had practically come to a stop, the mixture was deproteinized without admitting air, and the extract analysed for HMP and ∼ P (the energy-rich phosphate groups of ATP and ADP) by the spectrophotometric enzymic method (42), with results shown in table III.

The amount of ferricytochrome *c* reduced, calculated from the change of optical density, was in close agreement with the total amount of ferricytochrome *c* at the beginning of the experiment, and was also close to the amount of α-ketoglutarate disappearing, showing the absence of other reactions involving α-ketoglutarate or cytochrome *c*. The amount of phosphorylation is expressed as Δ HMP + Δ ∼ P, in order to allow for the phosphorylation of glucose by ADP, catalysed by myokinase + hexokinase,

$$\text{ADP} + \text{glucose} \rightarrow \text{HMP} + \text{AMP}$$

In this reaction, Δ HMP = —Δ ∼ P, so that Δ HMP + Δ ∼ P = 0. Thus it has no effect on the phosphorylation expressed as Δ HMP + Δ ∼ P.

There is no doubt that these experiments directly demonstrate phosphorylation with added ferricytochrome *c* as hydrogen-acceptor. Moreover, the fact that the P : αKg ratio is much greater than one and is sensitive to dinitrophenol (45) shows that phosphorylation in the respiratory chain is involved, as well as substrate-linked phosphorylation. The experiments show directly what there was good reason to expect, namely that at least one of the respiratory chain phosphorylative steps occurs between substrate and cytochrome *c*.

In less extensive experiments, we (31) have also shown phosphorylation coupled with the reduction of ferricytochrome *c* by succinate, and by β-hydroxybutyrate (see table IV). The P : 1/2 cytochrome *c* ratio with succinate, although low, was outside the possible experimental error, indicating a phosphorylative step between succinate and cytochrome *c*.

TABLE III.

*Phosphorylation coupled with the reduction of ferricytochrome c by α-ketoglutarate*

| | Ferricytochrome *c* reduced (μ-moles) |
|---|---|
| (i) Total amount used | 0.94 |
| (ii) Calculated from $\Delta\, d_{580}$ | 0.92 |
| 0 atom equivalent, using (i) | 0.47 |

| | Initial | Final | Δ |
|---|---|---|---|
| α Kg (μ-moles) | 0.98 | 0.53 | —0.45 |
| ∼P (μ-moles) | 0.59 | 0.43 | —0.16 |
| HMP (μ-moles) | 0.02 | 0.96 | 0.94 |

| | |
|---|---|
| Δ HMP + Δ∼P (μ-moles) | 0.78 |
| P : O | 1.67 |
| P : αKg | 1.74 |

See text for description of experiment.

Reaction mixture : rat-heart sarcosomes (isolated in 0.28 M sucrose, 0.01 M EDTA, pH 7.4), 0.47 mg. protein/ml. ; phosphate, pH 7.4, 0.03 M ; malonate, 0.01 M ; fluoride, 0.04 M ; AMP, $2 \times 10^{-4}$ M ; ADP, $2 \times 10^{-4}$ M ; α-ketoglutarate, $3.3 \times 10^{-4}$ M ; glucose, 0.02 M ; EDTA, 0.001 M ; magnesium, 0.005 M ; hexokinase, 151 units/mg. sarcosomal protein. Reaction vol., 3 ml. From Slater (45).

TABLE IV.

*Phosphorylation coupled with the reduction of ferricytochrome c by succinate and β-hydroxybutyrate*

| Substrate | Substrate → cyt. c | | | Substrate → $O_2$ | | |
|---|---|---|---|---|---|---|
| | ΔO | ΔP | P : O | ΔO | ΔP | P : O |
| Succinate . . . . . . | 0.57 | 0.10 | 0.18 | 1.43 | 1.37 | 0.96 |
| β-hydroxybutyrate . . | 0.27 | 0.34 | 1.27 | 0.37 | 0.78 | 2.12 |

Experiments carried out as in table III, using 0.06 M succinate or 0.06 M DL-β-hydroxybutyrate. No malonate (31).

Experiments of this type probably do not measure all the phosphorylation between substrate and cytochrome *c*. It is possible that added ferricytochrome *c* is reduced both directly by the preceding member of the respiratory chain (X) and through endogenous cytochrome *c*, thus :

$$\begin{array}{l} \quad\ \ \rightarrow \text{added cytochrome } c \\ \quad\ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \uparrow \\ \rightarrow X \rightarrow \text{endogenous cytochrome } c \end{array}$$

and that the reaction between X and endogenous cytochrome *c* is coupled with phosphorylation, but that between X and added cytochrome *c* is non-phosphorylative.

### *Cytochrome* c → $O_2$

The first indication that there might be phosphorylation in this reaction was obtained by Lehninger (4, 36), who studied the oxidation of a number of reducing agents by liver mitochondria, in the presence of cytochrome *c* and $^{32}P$. These agents directly reduce the cytochrome *c* which is then oxidized by the cytochrome *c* oxidase in the mitochondria. The only reducing agent to give rise to any incorporation of $^{32}P$ in ATP was ascorbate (and this was very small), and Lehninger recognized the possibility that the dehydroascorbate formed might give rise to other metabolites yielding oxidative phosphorylation. The negative results with most of the other reducing agents, including *p*-phenylenediamine, were readily explained, since these substances, or their oxidation products, inhibited phosphorylation with normal intermediary metabolites as substrate. The failure to obtain phosphorylation with cysteine could not, however, be explained so easily, and Lehninger felt that these experiments had some validity in ruling out the cytochrome oxidase reaction as a site of phosphate esterification (36).

Judah (46), in 1951, was the first to find a net uptake of phosphorus coupled with the oxidation of ascorbate (P : O = 0.50-0.91). Since the oxygen uptake (μ-atoms) agreed closely with the disappearance of ascorbate (μ-moles), Judah concluded that the further oxidation of dehydroascorbate was not responsible for the phosphorylation observed. However, in agreement with Lehninger, he found no phosphorylation with cysteine, and cysteine did not affect the phosphorylation associated with the oxidation of β-hydroxybutyrate. Judah realised that his experiments did not establish that the phosphorylation occurred between cytochrome *c* and oxygen.

The discovery (47, 48) that, during the metal-catalysed oxidation of ascorbate, there is formed some substance (possibly a labile one-electron oxidation product) which could oxidize DPNH in the presence of a specific enzyme, raised further doubts that the phosphorylation obtained with ascorbate was really associated with the cytochrome oxidase reaction. It appeared possible that the labile oxidation product of ascorbate might be formed during its oxidation by ferricytochrome *c* and act as a hydrogen donor to the respiratory chain, thereby yielding phosphorylation associated with the reduction of cytochrome *c*, rather than with its oxidation. This possibility was, however, made unlikely by experiments carried out independently in three laboratories (49, 50, 22). It was found that antimycin, in concentrations which practically completely blocked the respiratory chain between substrate and cytochrome *c*, had only a slight effect on the phosphorylation with ascorbate. Our experiment with heart sarcosomes is shown in figure 2.

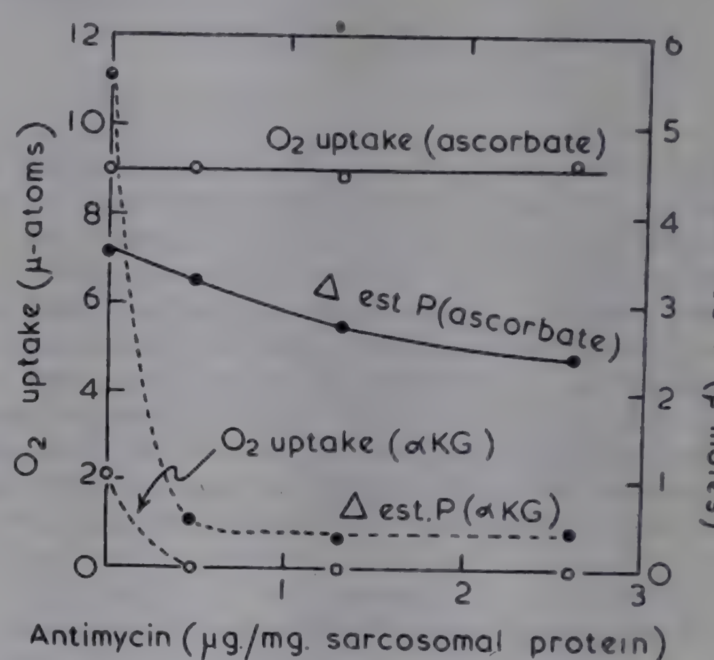



FIG. 2. — Effect of antimycin on the oxygen uptake and phosphorylation with ascorbate and α-ketoglutarate as substrates. Rat-heart sarcosomes isolated in 0.21 M sucrose, 0.01 M EDTA, pH 7.4, and given the hypotonic treatment described in ref. 52. Reaction mixtures : phosphate, pH 7.4, 0.03 M ; glucose, 0.03 M ; EDTA, 0.001 M ; fluoride, 0.04 M ; AMP = ADP, $10^{-3}$ M (ascorbate), or $6 \times 10^{-4}$ M (α-ketoglutarate) ; magnesium, 0.005 M ; malonate, 0.01 M (α-ketoglutarate only) ; hexokinase ; cytochrome *c*, $5.4 \times 10^{-5}$ M ; sarcosomes, 0.41 mg. protein/ml. ; ascorbate 0.01 M or α-ketoglutarate, 0.005 M. Reaction volumes, 3.0 ml. (ascorbate) or 1.0 ml. (α-ketoglutarate). The values for α-ketoglutarate have been multiplied 3-fold to allow for difference of volume. 30 min., 25° C.

I wish to thank Professor F. M. Strong for supplying the purified antimycin used in this experiment.

Phosphorylation coupled with the direct oxidation of ferrocytochrome *c*, in the absence of reducing agent, was first shown directly in 1954 by Nielsen and Lehninger (51). They used a low concentration of ferrocytochrome *c*, a very dilute mitochondrial suspension and measured the phosphorylation by determining the incorporation of $^{32}P$ into the organic phosphorus fraction

(table V). The P : 1/2 cytochrome *c* ratios found were about the same as the P : O ratios obtained with ascorbate and catalytic amounts of cytochrome *c*.

TABLE V.
*Phosphorylation coupled with the oxidation of ferrocytochrome* c

| | Expt. 1 | Expt. 2 | Expt. 3 | |
|---|---|---|---|---|
| | | | 2 min. | 15 min. |
| Ferrocytochrome *c* (μ-moles) | | | | |
| Initial | 0.0165 | 0.0165 | 0.0165 | 0.0165 |
| Final | 0.0103 | 0.0107 | 0.0145 | 0.0079 |
| Δ | 0.0062 | 0.0058 | 0.0020 | 0.0086 |
| Organic phosphate (μ-moles) | | | | |
| Δ | 0.0019 | 0.0025 | 0.0005 | 0.0019 |
| P : 1/2 cyt. *c* | 0.61 | 0.86 | 0.53 | 0.44 |

Nielsen and Lehninger's (51) experiments with hypotonically-treated liver mitochondria. Phosphate, 8.5 × $10^{-4}$ M; ADP, 1.5 × $10^{-3}$ M; TRIS, pH 7.4, 0.02 M; EDTA, 0.001 M; magnesium; antimycin, 0.02 μg. Reaction started by adding mitochondria derived from 0.5 mg. wet weight rat liver suspended in 0.075 M sucrose + 0.001 M EDTA. 23-26° C.

We have confirmed Lehninger's findings, using heart sarcosomes; by using much larger amounts of ferrocytochrome *c*, we were able to demonstrate a net phosphorylation (52). The results of one experiment are shown in table VI. The small amount of HMP found in the reference cell is mainly due to the non-oxidative phosphorylation of glucose by myokinase and hexokinase, and will also include phosphorylation derived from the oxidation of traces of substrates in the sarcosomal preparation. The HMP formed in cell 2 minus that in cell 1 represents the phosphorylation coupled

TABLE VI.
*Phosphorylation coupled with the oxidation of ferrocytochrome* c

| | Cell 1 | Cell 2 |
|---|---|---|
| Ferrocytochrome *c* (μ-moles) | | |
| Initial | 0 | 1.42 |
| Final | 0 | 0.32 |
| Δ | 0 | 1.10 |
| Hexosemonophosphate (μ-moles) | | |
| Initial | 0.022 | 0.022 |
| Final | 0.058 | 0.244 |
| Δ | 0.036 | 0.222 |
| Δ (corr.) | — | 0.186 |
| P : 1/2 cyt. *c* | — | 0.34 |

Slater's (52) experiments with hypotonically-treated heart sarcosomes. Sarcosomes isolated in 0.21 M sucrose, 0.01 M EDTA, pH 7.4, and suspended in this solution diluted 50-fold with water. Phosphate, pH 7.4, 0.03 M; glucose, 0.02 M; EDTA, $10^{-3}$ M; fluoride, 0.04 M; AMP, 2 × $10^{-4}$ M; ADP, 2 × $10^{-4}$ M; hexokinase, 127 units; $MgCl_2$, 0.005 M. 1.6 μ-moles cytochrome *c* (89 % reduced, 11 % oxidized) in cell 2 only. Reaction started by adding sarcosomes containing 0.19 mg. protein to each cell.

with the oxidation of ferrocytochrome *c*. The corrected value (0.186 μ-mole) is many times greater than the sensitivity of the analytical method (about 0.01 μ-mole), and corresponds to a P : 1/2 cytochrome *c* ratio of 0.34. When the same preparation oxidized ascorbate in the presence of catalytic amounts of cytochrome *c*, the P : O ratio was 0.32.

Nielsen and Lehninger's results and our own agree in showing about the same phosphorylation ratios with ferrocytochrome *c* and with ascorbate as primary hydrogen donors. It appears, then, to be established that phosphorylation can be coupled with the oxidation of ferrocytochrome *c* by mitochondrial or sarcosomal preparations, and that the phosphorylation formed with ascorbate is indeed associated with the cytochrome oxidase reaction. Maley and Lardy (49) have shown that adrenaline or 3,4-dihydroxyphenylalanine may replace ascorbate in this system.

The actual P : O ratios are considerably higher in Lehninger's and Lardy's studies with liver mitochondria, than in ours with heart sarcosomes. Lehninger *et al.* (50) found a mean of 0.79 with ascorbate, while Maley and Lardy (49) obtained a mean of 0.76 with ascorbate and 0.75 with adrenaline. Our results (22) with ascorbate lie between 0.27 and 0.41, with a mean of 0.34 (14 experiments).

There is, however, a serious difficulty in measuring these ratios. Lehninger (50) found that it was necessary to treat liver mitochondria under hypotonic conditions in order to obtain the highest phosphorylation with ascorbate. Two of his experiments are plotted in figure 3, together with two of ours with heart sarcosomes.

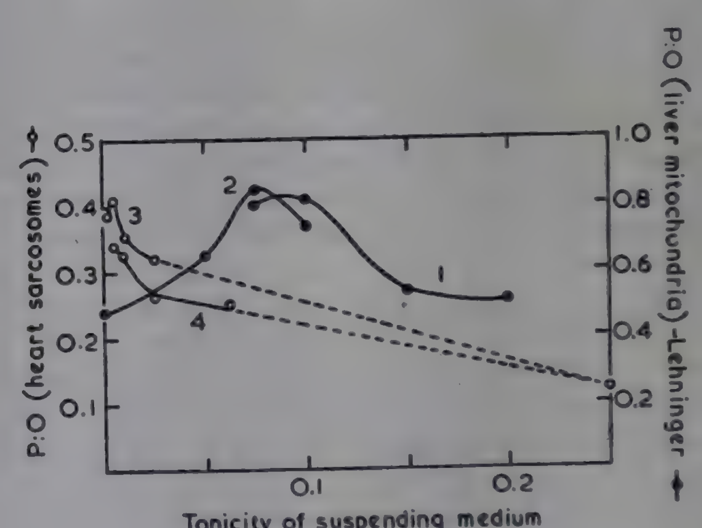



FIG. 3. — Effect of tonicity of suspending medium on P : O ratio, with ascorbate. Curves 1 and 2, two experiments of Lehninger *et al.* (50) with liver mitochondria; curves 3 and 4 two experiments of Slater (22) with heart sarcosomes.

It is clear that the heart sarcosomes require much lower tonicities for optimal ratios, and it is by no means certain that the full potential activity of the sarcosomes can be revealed in experiments of this type. Sarcosomes isolated and suspended in isotonic media have very low P : O ratios (0.08-0.15, mean 0.12 in 10 experiments). The high degree of hypotonicity necessary to invoke optimum phosphorylation with ascorbate has a variable effect on the ratios obtained with α-ketoglutarate

ubstrate. Sometimes there is little effect, but usually he P : O ratio dropped somewhat. The mean P : O atio of these treated sarcosomes was 2.50 (cf. table I). 'he hypotonic treatment markedly increased the rate f reduction of added cytochrome $c$ by α-ketoglutarate, nder anaerobic conditions, but had no effect on the mount of phosphorylation (45).

Surprisingly, Maley and Lardy's (49) high P : O ratios vith ascorbate were obtained with liver mitochondria solated and suspended in 0.25 M sucrose, without any ypotonic pretreatment.

The cytochrome oxidase reaction is probably :

$$\text{cyt. } c \rightarrow \text{cyt. } a \rightarrow \text{cyt. } a_3 \rightarrow O_2$$

If phosphorylation occurred between two cytochromes, we might expect a P : cytochrome $c$ ratio of 1, *i.e.* a P : O ratio of 2. This seems rather unlikely, and the highest P : O ratios are much less than 2. In the present state of our knowledge, it appears more likely that the phosphorylation is associated with the oxygen reaction :

$$4a_3^{++} + O_2 + 4H^+ \rightarrow 4a_3^{+++} + 2H_2O$$

If this reaction takes place in four separate single electron steps, the P : O ratios found by Lardy and Lehninger, corresponding to a P : $O_2$ ratio of 1.5, would suggest that at least two of these steps are phosphorylative. However, it is possible that two of the four oxidizing equivalents of the $O_2$ molecule are transferred simultaneously in the one reaction (*e.g.* George (52*a*)), and that the phosphorylation occurs in this reaction.

*Experiments with artificial hydrogen-acceptors*

Artificial hydrogen-acceptors have not proved very useful in localizing the phosphorylating steps. Dyestuffs with useful potentials, such as methylene blue, are inhibitors of oxidative phosphorylation. Some phosphorylation has been demonstrated with ferricyanide (53), but this has such a high potential that it is not possible to determine at which point in the respiratory chain it acts (6). Copenhaver and Lardy (18) have, however, found some phosphorylation in the reaction between β-hydroxybutyrate and ferricyanide in the presence of antimycin, suggesting that one of the steps between DPNH and the antimycin-sensitive factor is phosphorylative.

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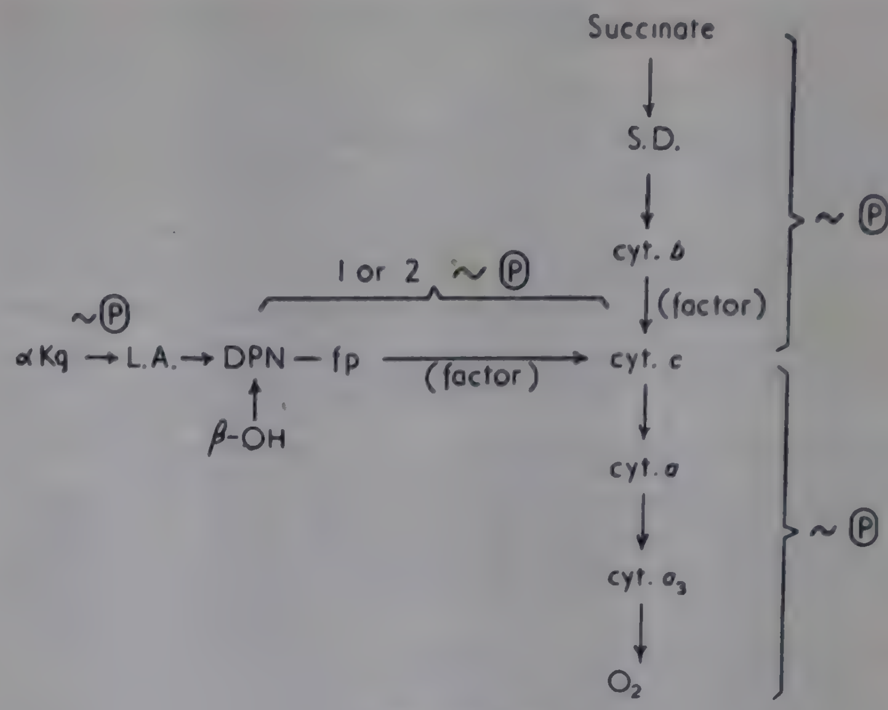
On the basis of these experiments, the phosphorylative steps in the respiratory chain may be located as in scheme E. The number of phosphorylative steps need not be the same as the P : O ratio, which might be decreased by side-reactions. The only single step which has been definitely identified as phosphorylative is the reaction between α-ketoglutarate and α-lipoic acid. The reaction between DPNH and flavoprotein has often been thought of as a phosphorylating reaction, and this may be what Copenhaver and Lardy studied in their reaction β-hydroxybutyrate → ferricyanide, in the presence of antimycin, but there is no direct evidence. The possible role of cytochrome $b$ is discussed below.




Scheme E

*Fractionation of the enzymes involved in oxidative phosphorylation*

When mitochondria or sarcosomes are mechanically damaged, particularly after they have been allowed to swell by suspension in a hypotonic medium, they disrupt into small particles, which may be collected by high-speed centrifugation or acidification to pH 5.7. These particles actively oxidize succinate and DPNH, but are devoid of many of the enzymes which are present in the intact mitochondria or sarcosomes, for example fumarase. They are also incapable of carrying out oxidative phosphorylation (the P : O ratio with succinate is less than 0.005). We have suggested that the sarcosomal fragments which constitute the Keilin and Hartree preparation are derived from the sarcosomal membrane and that the phosphorylating and some other enzymes are contained inside the sarcosome in a relatively soluble form and are washed away when the sarcosome is disrupted (54). No success in making these sarcosomal fragments phosphorylate by the addition of soluble enzymes or coenzymes has yet been reported.

Green (27) subjected sarcosomes to a milder treatment and obtained particles which oxidized succinate, but none of the other members of the tricarboxylic acid cycle. The oxidation of succinate to fumarate was accompanied by some phosphorylation, which a P : O ratio of 0.38. It appears that this preparation, which probably represents an intermediate stage between the intact sarcosome and the sarcosomal fragments in the Keilin and Hartree heart-muscle preparations, has retained some phosphorylating enzymes, although many dehydrogenases have been lost or inactivated.

Pinchot (55) has separated a sonic extract of *Alcaligenes fœcalis* into three components, a particulate DPNH oxidase system, a soluble protein fraction and a heat-stable non-dialysable component present in the boiled extract. The addition of the soluble protein fraction stimulated the rate of oxidation by the oxidase preparation, but phosphorylation required the further addition of the boiled extract. The P : O ratio was rather

low (0.3), but the extracts contained much ATP-ase and myokinase.

Tissières and I (56) have made some preliminary experiments on the phosphorylation in cell-free extracts of *Azotobacter vinelandii* (*cf.* 57). In table VII, fraction $P$ consists of particles not sedimented by centrifugation at 12 000 R.P.M. (9500 $g$), but sedimented at about 120 000 $g$ for 90 min. Fraction $S$ is the clear supernatant taken from the top of the tube after the latter centrifugation. The small particles in fraction $P$ oxidized succinate with a P : O ratio approaching 1 in experiment 1 (the ratio was often lower, *e.g.* experiment 2). Fraction $S$, which contains proteins of molecular weight less than $10^6$, possesses considerable 'soluble' succinic oxidase activity (*cf.* 58), especially in experiment 1, with an appreciable phosphorylation. When the two fractions were mixed, both the oxygen uptake and the phosphorylation were stimulated, far beyond that expected from an additive effect, suggesting that some separation of enzymes concerned might have been achieved.

TABLE VII.

*Oxidative phosphorylation in extracts of* Azotobacter vinelandii

| Expt. | Fraction | Protein (mg.) | $O_2$ uptake (μ-atoms) | Δesterified P (μ-moles) | P : O |
|---|---|---|---|---|---|
| 1 | $P$ | 0.35 | 1.37 | 1.19 | 0.87 |
| | $S$ | 9.3 | 3.61 | 0.56 | 0.16 |
| | $P + S$ | 9.65 | 11.9 | 5.42 | 0.455 |
| 2 | $P$ | 1.5 | 2.10 | 0.49 | 0.23 |
| | $S$ | 12 | 0.67 | 0.22 | 0.33 |
| | $P + S$ | 13.5 | 6.94 | 2.32 | 0.33 |

The extract was prepared by grinding the cells with glass powder, followed by water extraction. The mixture was centrifuged at 7 100 r.p.m. (4 000 $g$) for 15 min., and the supernatant clarified by successive centrifugations at 8 100 R.P.M. (4 500 $g$) for 10 min., and at 11 900 R.P.M. (9 500 $g$) for 10 min. in a refrigerated centrifuge. The supernatant (v ml.) was centrifuged at 40 000 r.p.m. (about 120 000 $g$) for 90 min. in a model L Spinco centrifuge, and the sediment suspended in 0.25 v ml. 0.05 M phosphate buffer, pH 7.4, to give fraction $P$. Fraction $S$ was taken from the top part of the supernatant.

The reaction mixture was : succinate, 0.02 M ; ADP, $6 \times 10^{-4}$ M ; AMP, $6 \times 10^{-4}$ M ; glucose, 0.03 M ; phosphate, 0.03 M ; EDTA, 0.001 M ; magnesium. 0.005 M ; hexokinase, 274 units.

Fraction $P$ did not oxidize α-ketoglutarate unless $S$ was added ; the P : O ratio with the two fractions together was 0.82.

*Mechanism of generation of energy-rich bonds*

Lipmann (33), in 1946, suggested that phosphorylation linked with the reaction between two adjacent members of the respiratory chain occurred by the mediation of a third substance, for example :

$$AH_2 + C \rightarrow A + CH_2$$

$$CH_2 + H_3PO_4 \rightarrow CH_2\text{-}H_2PO_3 \text{ } (CH_2 \text{ may be first dehydrated})$$

$$CH_2\text{-}H_2PO_3 + B \rightarrow C \sim H_2PO_3 + BH_2$$

$$C \sim H_2PO_3 + \text{ADP} \rightarrow C + \text{ATP}$$

where $AH_2$ and $B$ are adjacent members of the respiratory chain, and $C$ is the third substance. Actually, since $C$ undergoes oxido-reduction, it could be considered a member of the respiratory chain, and the last three equations describe a mechanism of the phosphorylation between $CH_2$ and $B$. In any case, it is clear that a different mediator is required at each phosphorylative step in the respiratory chain, since the oxidation-reduction potential of $C \rightleftarrows CH_2$ must be suitably related to those of the $A \rightleftarrows AH_2$ and $B \rightleftarrows BH_2$ systems. This means that a phosphorylative respiratory chain will have two or three more members than a non-phosphorylative chain. This is implied in the recent scheme of Martius (59).

Developments over the last five years in the mechanism of substrate-linked phosphorylation, initiated particularly by Racker (60), have suggested an alternative possibility (61), represented by the equations :

$$AH_2 + B + C \rightleftarrows A \sim C + BH_2 \qquad (1)$$

$$A \sim C + H_3PO_4 + \text{ADP} \rightleftarrows A + C + \text{ATP} \qquad (2)$$

The essential differences between this scheme and Lipmann's are firstly, $C$ does not undergo oxido-reduction (compare coenzyme A) and the same $C$ could conceivably act at each phosphorylative step ; secondly, the energy-rich link is formed prior to the introduction of phosphate, as in the formation of acetyl- or succinyl-coenzyme A. This is suggested by the fact that phosphate is not obligatory for respiration in mitochondria in the presence of dinitrophenol (62), or in sarcosomal fragments which do not phosphorylate (63, 64).

These equations show that for respiration to proceed, $A \sim C$ must be broken down to $A$ and $C$. In the phosphorylating intact mitochondria, this is brought about by reaction (*2*) ; respiration will, therefore, cease when all the ADP is phosphorylated (or all the phosphate is used up), as has been found to be the case by a number of workers, notably Lardy and Wellman (65). Respiration will proceed again if the ATP is dephosphorylated to ADP, *e.g.* by the addition of hexokinase-glucose or ATP-ase. Respiration is also restored by the addition of dinitrophenol, which opens up another pathway for the splitting of $A \sim C$, not involving ADP or $H_3PO_4$, perhaps a hydrolysis (66, 67).

$$A \sim C + H_2O \rightarrow A + C \qquad (3)$$

In the presence of hexokinase and glucose, the rate of respiration will depend upon the activities of the enzyme systems catalysing reactions (*1*) and (*2*). If reaction (*2*) can proceed much more rapidly than reaction (*1*), the increased rate of breakdown of $A \sim C$ brought about by the introduction of reaction (*3*) into the system will have little effect on the overall reaction. This agrees with the effect of dinitrophenol on the rate of respiration of actively phosphorylating systems, such as liver mitochondria (65) or heart sarcosomes (68). The substitution of reaction (*3*) for reaction (*2*) will, however, prevent the synthesis of ATP. If the rate of reaction (*1*) is limited by the rate of breakdown of $A \sim C$, the addition of dinitrophenol might be expected to increase the rate of respiration, even in the presence of hexokinase and glucose, and this has been found to be the case with

some preparations of blowfly sarcosomes (68), mammary gland homogenates (69) and with hamster liver mitochondria in the presence of thyroxine (70).

It is unlikely that reactions (*1*) and (*2*) take place as written; separate bimolecular reactions are probably involved. Boyer *et al.* (71) have found that liver mitochondria in the absence of substrate catalyse a rapid exchange of the P atom of inorganic phosphate with ATP, accompanied by a 20 times more rapid exchange of the oxygen atoms of inorganic phosphate with those of water (see also 72). This could be explained on the basis of the above scheme by breaking reaction (*2*) into :

$$A \sim C + H_3PO_4 \rightleftarrows A + C \sim H_2PO_3 \text{ (or } A \sim H_2PO_3 + C) \qquad (2a)$$

$$C \sim H_2PO_3 \text{ (or } A \sim H_2PO_3) + ADP \rightleftarrows C \text{ (or } A) + ATP \qquad (2b)$$

Reaction (*2a*) could explain the exchange between the O atoms of phosphate and water, while the two reactions together would explain the exchange of the P atoms of $H_3PO_4$ and ATP. The relative rates of the two exchanges suggest that reaction (*2a*) is much faster than reaction (*2b*). As would be expected from the above discussion, the exchange reactions were abolished by dinitrophenol (71).

It is also apparent that so far as the above discussion is concerned, reaction (*1*) could just as well be written :

$$AH_2 + B + C \rightleftarrows A + BH_2 \sim C \qquad (1')$$

Chance (14) has evidence in favour of this variation.

The high proportion of lipide in mitochondria, sarcosomes and sarcosomal fragments raises the possibility that the hypothetical *C* is a lipide-soluble compound. The fact that the water-soluble vitamins often form part of respiratory enzyme systems naturally suggests that the fat-soluble vitamins might also be involved (see, however, Kodicek, 73). Weil-Malherbe (74) pointed out that the symptoms of vitamin E-deficiency were suggestive of a decreased ability of the muscles to carry out oxidative phosphorylation (see 75). However, Rabinowitz and Boyer (76) found no difference between the phosphorylative activity of normal and dystrophic hearts. Martius (59) has mentioned that a positive effect of the administration of vitamin E was obtained in experiments with rats and golden hamsters, but no details have been given.

There appears to be little information on the distribution of the fat-soluble vitamins within the cell. Isolated liver mitochondria contain 3 μ-moles vitamin A/g. protein (77), but as liver stores large amounts of the fat-soluble vitamins, this may not be very significant. Dr. E. Kodicek (78) has kindly examined the heart sarcosomal fragments for vitamin D. None could be detected, and it was concluded that there was less than 0.03 μ-mole vitamin D/g. sarcosomal protein, which is small compared with the content of other respiratory catalysts, *e.g.* cytochrome *c*, 0.8 μ-mole/g. protein (79). It is easier for us to prepare the large amounts required for these analyses in the form of sarcosomal fragments, rather than the intact sarcosome. It would be expected that lipide-soluble materials present in the intact sarcosome would be retained in the fragments. No values for the vitamin E and K contents of the sarcosomal fragments are yet available.

### *Role of vitamin K*

Martius and Nitz-Litzow have brought forward the following evidence in favour of the view that vitamin K is concerned in oxidative phosphorylation.

— Dicoumarol and other substances chemically related to vitamin K uncouple phosphorylation associated with the oxidation of β-hydroxybutyrate by liver mitochondria (80).

— Mitochondria isolated from the livers of vitamin K-deficient chicks give lower P : O ratios than normal mitochondria, with β-hydroxybutyrate as substrate. Phosphorylation coupled with the oxidation of succinate was little affected (81).

— The addition of vitamin $K_1$ to the mitochondria from deficient chicks raised the P : O ratio to normal (82).

Martius (15) places vitamin $K_1$ in the respiratory chain, as shown in scheme D.

### *Role of manganese*

It has long been recognized that many of the oxidative activities in tissue preparations and most of the phosphorylative are very labile. We found that this instability, in heart preparations at least, is associated with the presence of calcium in the sarcosomes (83, 84). The calcium becomes bound during the isolation of the sarcosomes and this can be prevented by including 0.01 M ethylenediaminetetraacetate (EDTA) in the isolation medium. Sarcosomes prepared in this way are much more stable (see table VIII).

Associated with the loss of enzyme activity is a swelling of the sarcosome which was studied microscopically and, more quantitatively, by measuring the degree of light scatter (85, 86, 87). This swelling which occurs in isotonic media is greatly slowed by the addition of EDTA. In the absence of EDTA, a considerable protection of the enzyme activity (see table VIII) and a decrease of the rate of swelling was obtained by the

TABLE VIII.

*Effect of incubation of heart sarcosomes on oxidative and phosphorylative activities, and protective effect of EDTA and ADP*

| Incubation time (min.) | Addition during incubation | Substrate | $Q_{O_2}$ | P : O |
|---|---|---|---|---|
| 0 | — | α-ketoglutarate | 95 | 2.50 |
| 15 | None | » | 28 | 2.00 |
| 15 | EDTA(0.01 M) | » | 112 | 2.46 |
| 15 | ADP(0.003 M) | » | 74 | 2.31 |
| 0 | — | Succinate | 278 | 1.01 |
| 15 | None | » | 465 | 0.73 |
| 15 | EDTA(0.01 M) | » | 278 | 1.11 |
| 15 | ADP(0.003 M) | » | 273 | 0.88 |

Incubation at 25° C. From Slater and Cleland (84).

addition of ADP or ATP. Even in the presence of EDTA, the addition of ATP further decreased the rate of swelling (86).

Lindberg and Ernster (88) have shown that manganese can prevent the inactivation of the oxidation of glutamate by liver mitochondria caused by incubation of the latter with calcium and hexokinase. Moreover, they were able to restore both the respiration and the phosphorylation by the addition of manganese + ATP + DPN to a system inactivated in this way (table IX). Lindberg and Ernster concluded that manganese is a co-factor

TABLE IX.

*Effect of manganese, ATP and DPN on mitochondrial respiration inhibited by calcium*

| Added at zero time | Added after 12 min. incubation (30° C.) | $O_2$ uptake (*) (μ-atoms) | P uptake (*) (μ-moles) |
|---|---|---|---|
| — | — | 22.7 | 53.5 |
| $Ca^{++}$ | — | 0.8 | — |
| $Ca^{++}$ | $Mn^{++}$ | 2.3 | — |
| $Ca^{++}$ | ATP | 1.2 | — |
| $Ca^{++}$ | DPN | 15.0 | 1.2 |
| $Ca^{++}$ | $Mn^{++}$ + ATP | 0.7 | — |
| $Ca^{++}$ | $Mn^{++}$ + DPN | 16.9 | 22.6 |
| $Ca^{++}$ | ATP + DPN | 15.8 | 2.1 |
| $Ca^{++}$ | $Mn^{++}$ + ATP + DPN | 20.7 | 49.5 |

(*) Between 12 and 37 min. From Lindberg and Ernster (88).

of oxidative phosphorylation and that EDTA might, besides binding calcium, also remove the manganese from the mitochondria. If this were so, one might expect that isolation of the sarcosomes in EDTA would decrease the phosphorylation ratios, which might be increased by the addition of manganese. This possibility has been tested in the experiments which are summarized in table X.

Three types of preparations were used. In experiment 1, table X, the sarcosomes were isolated and suspended in 0.21 M sucrose, 0.01 M EDTA, pH 7.4, and the reaction mixture contained 0.001 M EDTA derived from the sarcosomal suspension. Since EDTA chelates with manganese very firmly (89), it is not surprising that there was no effect of added manganese until its total concentration exceeded that of the EDTA. The rate of oxidation was slightly increased and the phosphorylation ratios slightly decreased by the larger amounts of manganese.

In experiment 2, the sarcosomes were isolated in the same medium as in experiment 1, but were washed by sedimentation in the centrifuge with 0.25 M sucrose, free from EDTA. The reaction mixture was, therefore, practically free from EDTA. Under these conditions, $10^{-4}$ M $MnCl_2$ increased the rate of oxidation by nearly 50 %, while the phosphorylation ratios were increased very slightly from a somewhat low value to a figure typical of preparations isolated and suspended in media containing EDTA. Similar results were obtained in experiment 3, in which a preparation, isolated in sucrose

TABLE X.

*Effect of added manganese on the rate of oxidation of α-ketoglutarate and on the phosphorylation ratios*

| Expt. | $Mn^{++}$ (mM) (Total) | $Mn^{++}$ (mM) (Free) | EDTA (mM) | $O_2$ uptake (μ-atoms) | —ΔαKg (μ-moles) | Δesterifieid P (μ-moles) | P : O | P : Kg |
|---|---|---|---|---|---|---|---|---|
| 1 | 0 | | 1 | 3.11 | 2.83 | 8.88 | 2.85 | 3.13 |
| | 1 | 0 | 1 | 3.02 | 2.78 | 8.45 | 2.80 | 3.04 |
| | 1.1 | 0.1 | 1 | 3.24 | 3.01 | 9.23 | 2.85 | 3.06 |
| | 1.2 | 0.2 | 1 | 3.47 | 3.34 | 9.58 | 2.76 | 2.87 |
| | 1.5 | 0.5 | 1 | 3.70 | 3.24 | 9.55 | 2.58 | 2.95 |
| | 2.0 | 1.0 | 1 | 3.53 | 3.20 | 9.13 | 2.59 | 2.86 |
| 2 | 0 | 0 | 0 | 2.29 | 2.22 | 6.03 | 2.63 | 2.72 |
| | 0.1 | 0.1 | 0 | 3.25 | 3.10 | 9.23 | 2.84 | 2.98 |
| | 0.5 | 0.5 | 0 | 3.26 | 3.13 | 9.21 | 2.82 | 2.94 |
| | 1.0 | 1.0 | 0 | 3.08 | 3.11 | 8.85 | 2.87 | 2.85 |
| | 2.0 | 2.0 | 0 | 3.22 | 3.03 | 8.48 | 2.63 | 2.80 |
| 3 | 0 | 0 | 0 | 2.44 | 2.20 | 6.15 | 2.52 | 2.80 |
| | 0.01 | 0.01 | 0 | 2.86 | 2.39 | 7.10 | 2.48 | 2.97 |
| | 0.1 | 0.1 | 0 | 3.52 | 2.90 | 9.9 | 2.81 | 3.41 |
| | 1.0 | 1.0 | 0 | 3.77 | 3.25 | 10.0 | 2.65 | 3.08 |
| | 0 | 0 | 2 | 2.76 | 2.28 | 7.42 | 2.70 | 3.25 |
| | 1.0 | 0 | 2 | 2.68 | 2.39 | 7.04 | 2.63 | 2.95 |

Sarcosomal preparations: exptl. 1, isolated and suspended in 0.21 M sucrose, 0.01 M EDTA, pH 7.4; expt. 2, isolated in same medium and sarcosomes washed, and suspended in 0.25 M sucrose; expt. 3, isolated and suspended in 0.23 M sucrose, 0.01 M tris (hydroxymethyl)-aminomethane, pH 7.2. 25° C., 30 min. [Free $Mn^{++}$] = [Total $Mn^{++}$] — [EDTA]. Reaction mixture: phosphate, pH 7.4, 0.03 M; AMP, $6 \times 10^{-4}$ M; ADP, $6 \times 10^{-4}$ M; glucose, 0.03 M; α-ketoglutarate, $5.5 \times 10^{-3}$ M; malonate, 0.01 M; fluoride, 0.04 M; cytochrome c, $3.4 \times 10^{-5}$ M; magnesium, 0.005 M; sarcosomes, 0.6-0.8 mg./ml.; hexokinase, 130 units.

containing 0.01 M tri(hydroxymethyl)aminomethane as buffer, was used.

These results are not what would be expected if manganese were a co-factor of oxidative phosphorylation, and is removed by isolation of the sarcosomes in media containing EDTA. Under our conditions, the effect of manganese was primarily on the oxygen uptake and the largest effects were obtained with preparations made without EDTA.

Although Lindberg and Ernster have clearly shown that manganese (in the presence of ATP and DPN) can reactivate preparations which have been inactivated by incubation in the presence of calcium, this does not prove that manganese is directly involved in the enzyme systems of the initially active preparation. It is possible that manganese reverses the inactivation in a more indirect manner, for example it may assist the ATP to bring about the extrusion of water from the mitochondria (90, 91). The effect of cobalt on brain mitochondria (92) should also be considered in this connexion.

Lindberg and Ernster (93) have also shown that the addition of ATP can restore the phosphorylation associated with the oxidation of succinate, after incubation with calcium. Manganese was not essential, but much less ATP was required for the reactivation in its presence. In similar experiments to those described in table X, we have found no stimulation of the P : O ratio by the addition of manganese (31). In fact, there was a progressive lowering of the ratio with increasing quantities of manganese.

### *Role of cytochrome* b

Chance and Williams (14) have found that the addition of ADP to rat liver mitochondria in the presence of oxygen and substrate caused the DPNH, flavoprotein, cytochrome *b* and *c* in the mitochondria to become more oxidized and the cytochrome *a* to become more reduced. When the ADP became completely phosphorylated by oxidative phosphorylation, these changes in the steady-state oxido-reduction were reversed and the cycle could be repeated by a second addition of ADP.

Holton (94) has found that, in heart sarcosomes, cytochrome *b* behaves in the opposite way, *i.e.* it becomes more oxidized when the ADP is phosphorylated. This effect is specific for cytochrome *b* among the cytochromes, but Holton was unable to test possible effects on DPN or flavoprotein.

Holton's methods were similar to those used by Chance, but were carried out with a commercially available spectrophotometer, which was found to have sufficient sensitivity and stability. In a suspension made anaerobic by allowing succinate to consume oxygen in the presence of ADP, magnesium, EDTA and isotonic sucrose, the whole cytochrome system was completely reduced. When oxygen was stirred into the solution, the extinction at 445 mμ rapidly decreased as cytochrome $a_3$ was oxidized, and stayed at a constant value for some time (see figure 4A). When all the oxygen was consumed, the cytochrome was again reduced, resulting in an increase of the extinction. Similar changes occurred at 420 mμ, where the kinetics of cytochrome *c* can be studied. At 430-435 mμ, and also at 560-565 mμ, where reduced cytochrome *b* has absorption peaks, the immediate drop of the extinction corresponded to only partial oxidation of the cytochrome *b*, and the extinction continued to decrease during the period corresponding to the aerobic steady-state at other wave-lengths. This change of the

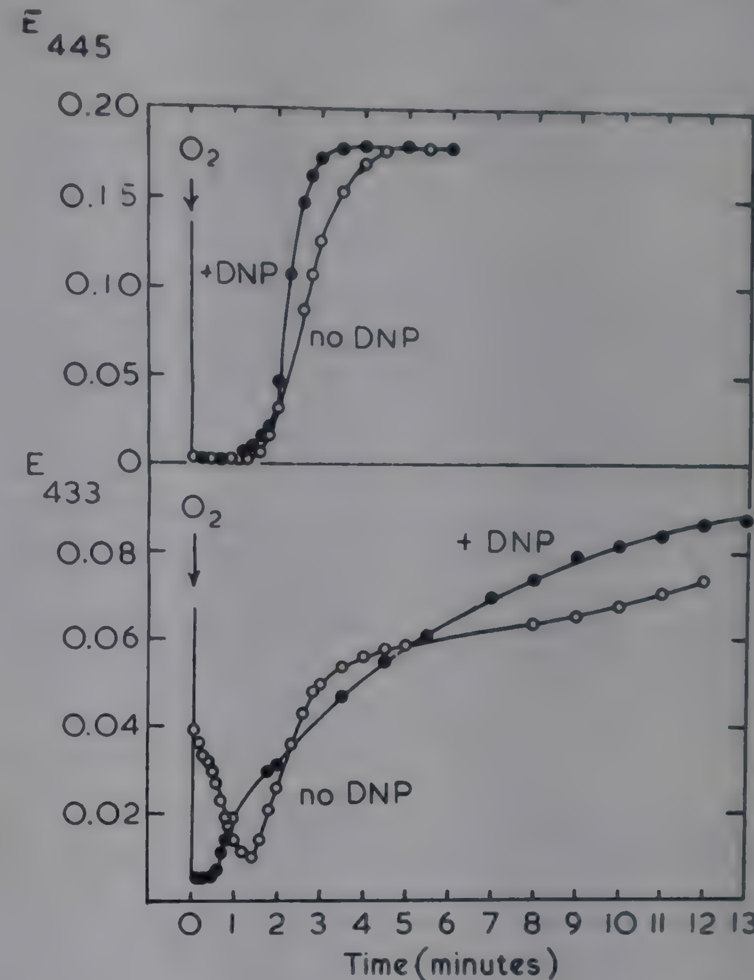



FIG. 4. — Oxidation of cytochrome $a_3$ (445 mμ) and cytochrome *b* (433 mμ) on the addition of oxygen to a sarcosomal suspension and the subsequent reduction when the suspension becomes anaerobic, and the effect of dinitrophenol (DNP). No significance should be attached to the different times at which reduction commences, since this depends upon the amount of oxygen added, which was not the same in different experiments. O——O, no DNP; ●——●, 8.4 × $10^{-5}$ M DNP. Optical depth, 0.5 cm. Rat-heart sarcosomes, 4 mg. protein/ml.; magnesium, 0.004 M; ADP, 1.7 × $10^{-4}$ M; succinate, 1.7 × $10^{-2}$ M; malonate, 0.0135 M (in absence of DNP) or 0.0084 M (in presence of DNP). Temperature, 14° C. Suspensions initially anerobic and aerated at zero time. From Holton (94).

steady-state of cytochrome *b* is shown more clearly in another experiment in figure 5. In this case, the extinction remained almost steady, corresponding to a high degree of reduction in the steady-state, for some time before the cytochrome *b* became more oxidized.

If ADP were omitted (figure 5), or if dinitrophenol was added (figure 4B), or if the Keilin and Hartree heart-muscle preparation was used instead of the sarcosomes, the abnormal initial kinetics at 430-435 mμ disappeared. The extinction fell very rapidly to a value which remained constant during the aerobic steady-state. The addition of dinitrophenol to the sarcosome preparation had no effect on the magnitude or kinetics of the changes of extinction observed at 445 mμ (cytochrome $a_3$) (figure 4A) or at 420 mμ (cytochrome *c*). Similarly, the omission of ADP had no effect at 445 mμ.

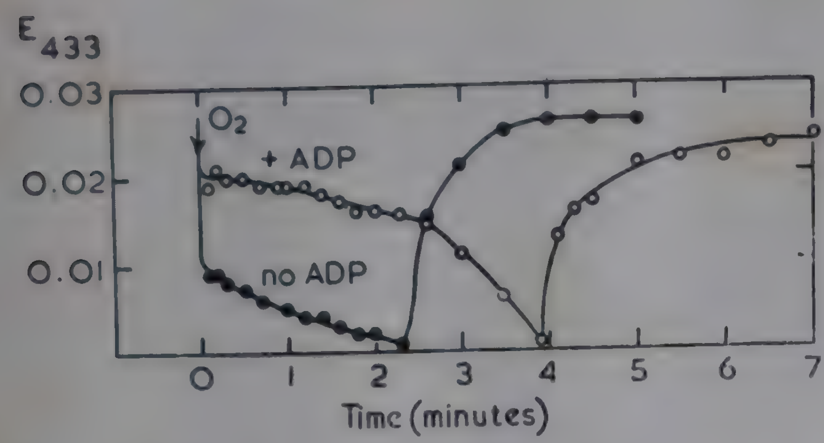



FIG. 5. — Effect of ADP on the kinetics of oxidation and reduction of cytochrome *b* in rat-heart sarcosomes. Optical depth, 0.1 cm. Succinate, 0.013 M; no $MgCl_2$ or malonate. o——o, $1.1 \times 10^{-4}$ M ADP; ●——●, no ADP. At zero time, freshly oxygenated samples of the suspensions at 2° C. were placed in the optical cell at room temperature. From Holton (94).

The most likely interpretation of Holton's experiments is that, in the presence of ADP, cytochrome *b* is mainly reduced, and becomes more oxidized when all the ADP is phosphorylated to ATP. This suggests that ADP is required for the rapid reduction of cytochrome *b*, thus :

$$\text{succinate} + 2b^{+++} + \text{ADP} + H_3PO_4 \rightleftarrows \text{fumarate} + 2b^{++} + \text{ATP}$$

but there are serious thermodynamic difficulties, since the equilibrium of this reaction lies far to the left. This simple mechanism also does not explain the action of dinitrophenol, which has the opposite effect from the addition of phosphate acceptor. So far as respiration is concerned, dinitrophenol and phosphate acceptor act in the same direction (65). However, Holton's results are consistent with Lundergardh's (95) finding with wheat roots that dinitrophenol markedly inhibited the rate of reduction of cytochrome *b*.

The difference between the results of Chance and Williams (14) and those of Holton (94) is probably due to the fact that the former authors used liver mitochondria, while Holton used heart sarcosomes. Although the respiration of the latter is markedly increased by a phosphate acceptor (17), the effect is less marked than with liver mitochondria (65). Thus the respiratory chain in liver mitochondria is almost completely blocked in the absence of ADP, and the effect of ADP on the cytochrome kinetics will be dominated by its effect on this block. In heart sarcosomes, where this block may be less effective, the effect of ADP on the reduction of cytochrome *b* is revealed.

. . .

Although none of the phosphorylative steps in the respiratory chain have been specifically identified, the possibilities have been considerably narrowed by work over the past six years, using isolated components of the respiratory chain as hydrogen or electron-donors (Lehninger) or as acceptors. It can now be concluded, with some degree of confidence, that phosphorylation occurs between (i) succinate and cytochrome *c*; (ii) cytochrome *c* and oxygen; (iii) DPNH and cytochrome *c* (there are probably two phosphorylative steps in this reaction).

However, our knowledge of the mechanism of the phosphorylation is still rudimentary. The scheme discussed in this paper is useful for describing, in general terms, the effects of phosphate acceptors and uncoupling agents, but it does not penetrate very deeply into the problem. Much more detailed information about the reactions in the respiratory chain is required. This may come from intensive chemical studies of portions of the respiratory chain in the mitochondria, *e.g.* the cytochrome oxidase reaction, or from fractionation studies of the type which have been so useful in elucidating the mechanism of the generation of energy-rich bonds at the substrate level.

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# Carbamyl phosphate

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(*Received* 4 *April* 1955)

During recent studies on citrulline synthesis (1) a new phosphorylated compound, carbamyl phosphate, was found to be an intermediary of some biological importance, mainly as carbamyl donor in a variety of enzymatic systems. The compound could be synthesized with surprising and gratifying ease. It forms spontaneously in an aqueous solution of cyanate and dihydrogen phosphate. For example, if a molar solution of the two potassium salts is kept at 30° C. for half an hour, about 50 % of the phosphate is in the form of the condensation product, carbamyl phosphate. On longer standing no more product is formed. The compound is easily isolated by alcohol fractionation as the water-soluble dilithium salt and has been used as such. It behaves similarly to carboxyl phosphates except that it is not split molybdate, a reaction which is so characteristic for all carboxyl phosphates as well as for creatine phosphate. In this respect it resembles arginine phosphate which, likewise, is stable to molybdate. Methodologically this stability is important since it permits the determination of formation or disappearance of carbamyl phosphate directly by the use of the Fiske and Subbarow procedure. For the determination of the orthophosphate content of the preparation freshly prepared reducing reagent is required for full color development in 5 minutes. It is necessary to read the tubes no later than 5 minutes after the addition of the acid molybdate reagent because of the slow hydrolysis of carbamyl phosphate in acid solution. The stability of carbamyl phosphate in acid and alkali closely resembles that of the carboxyl phosphates. In aqueous solution it decomposes at a rate similar to that of carboxyl phosphates; at 37° C. its half life is about 50 minutes; at 0° C. it is much more stable.

For estimation of carbamyl phosphate cold trichloroacetic acid is added and the protein precipitate centrifuged off in the cold. From the supernatant solution equal aliquots are introduced into two tubes, one of which is used immediately for the Fiske and Subbarow phosphate determination, taking the reading 5 minutes after addition of the acid molybdate reagent. The second sample is heated in 1 ml. of 0.01 N HCl for 1 minute at 100° C. by dipping into a boiling water bath and cooling afterwards. The difference may be considered carbamyl phosphate. In special cases, with much ATP(**) and little CAP, the greater instability of carbamyl phosphate towards 0.1 N alkali may be used for differentiation. The alkaline hydrolysis is carried out at room temperature and is complete in 10 minutes.

The hydrolysis of carbamyl phosphate is dependent on pH as shown in fig. 1. At pH 13 decomposition is very rapid and complete to inorganic phosphate and carbamic acid. On the other hand, at pH 1.5 the decomposition is not so rapid and the carbamic acid

THE HYDROLYSIS OF CARBAMYL PHOSPHATE

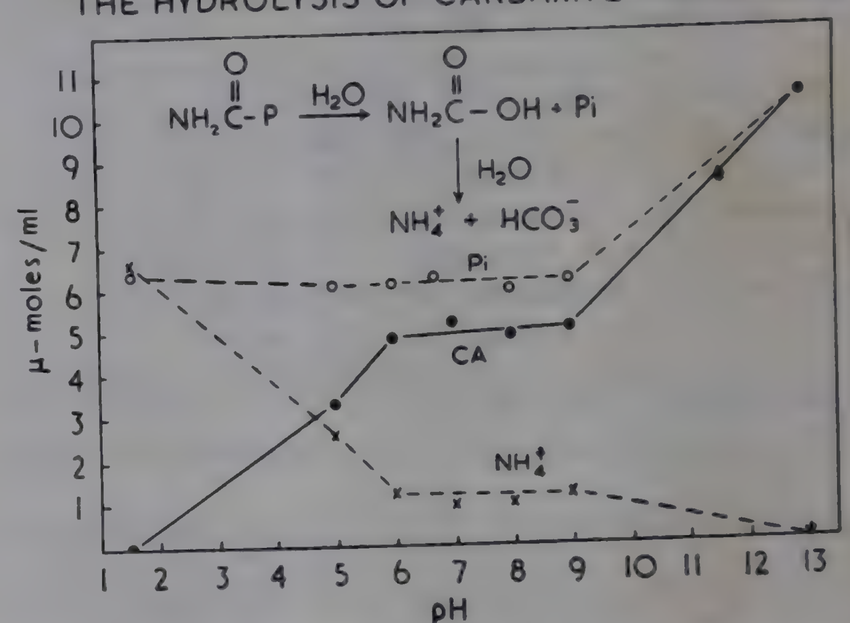


FIG. 1. — For pH 5 to pH 9 each ml. of solution contained : 11 micromoles of CAP and 100 micromoles each of acetate, imidazole, tris(hydroxymethyl)aminomethane and glycine adjusted to the indicated pH. For pH 1.5 and 13 the solutions were 0.05 M, HCl and KOH respectively, containing the same amount of CAP per ml. All vessels were incubated at 37° C. for 60 minutes.

formed is immediately hydrolyzed to ammonium bicarbonate. At intermediate pH levels the rate of phosphate release is comparable to that at the lower pH; however the carbamic acid formed is quite stable.

*The bacterial system of citrulline synthesis.* — The requirements for the arsenolysis and phosphorolysis of citrulline had suggested that the degradation of citrulline proceeded in two steps (2, 3, 4, 5, 6, 7, 8). It is now clear from the present studies that citrulline is synthesized and degraded in a two-step reaction. The first step is the phosphorylation of carbamate, the second is the carbamyl transfer from carbamyl phosphate to the acceptor ornithine :

$$H_2N\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH + ADP \sim O\text{-}PO_3^- \rightleftarrows H_2N\text{-}\overset{O}{\overset{\|}{C}} \sim O\text{-}PO_3^- + ADP \qquad (1)$$

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(**) The following abbreviations are used : ATP for adenosine triphosphate; ADP, adenosine diphosphate; and CAP, carbamyl phosphate.

$$H_2N\text{-}\overset{\overset{\displaystyle O}{\|}}{C} \sim O\text{-}PO_3^{=} + H_2N\text{-}(CH_2)_3\text{-}CHNH_2\text{-}COOH$$

$$\leftrightarrows H_2N\text{-}\overset{\overset{\displaystyle O}{\|}}{C} \sim NH\text{-}(CH_2)_3\text{-}CHNH_2\text{-}COOH + HO\text{-}PO_3^{=} \quad (2)$$

The same extract contains another carbamyl acceptor system; aspartic acid, as will be shown later, is converted to ureidosuccinic acid by reaction with carbamyl phosphate :

$$H_2N\text{-}\overset{\overset{\displaystyle O}{\|}}{C} \sim O\text{-}PO_3^{=} + HOOC\text{-}CH_2\text{-}\overset{\overset{\displaystyle NH_2}{|}}{C}H\text{-}COOH$$

$$\leftrightarrows HOOC\text{-}CH_2\text{-}\overset{\overset{\displaystyle NH \sim \overset{\overset{\displaystyle O}{\|}}{C}\text{-}NH_2}{|}}{C}H\text{-}COOH + HO\text{-}PO_3^{=} \quad (3)$$

Reaction 1) is more easily studied in the reverse direction by measuring the synthesis of ATP from carbamyl phosphate and ADP; the equilibrium appears to be greatly in favor of ATP formation. Accordingly, to form appreciable amounts of carbamyl phosphate, a phosphate donor of higher phosphate potential, *e.g.*, phosphoenol pyruvate, gives much better yields with $NH_3 + CO_2 \rightleftarrows$ (carbamate) than does ATP alone. There is, of course, no direct reaction with phosphoenol pyruvate, which acts only as a feeder system. A determination of the equilibrium between the components of this system is in progress.

Turning now to the carbamyl transfer in reaction 2), carbamyl phosphate with 1.3 equivalents of ornithine is completely converted to citrulline. The group potential of the carbamyl group of carbamyl phosphate, therefore, is considerably higher than that of the carbamyl group of citrulline. For this reason, ATP + carbamate in the presence of enzymes 1) and 2) are rather smoothly converted to citrulline. This, however, is also due to the presence of the second enzyme in very high concentration. In table I, the rates of the overall and partial reactions are compared and it appears that the carbamyl phosphate → citrulline reaction is by far the fastest. The carbamyl phosphate → ATP reaction, *i.e.*, 1) in reverse, is also still considerably faster than the overall reaction. From a comparison of the rates at 0° C. and 30° C. it may be seen that reaction 1), the reaction between ATP and carbamate, is the pacemaker, the overall reaction and reaction 1) showing similar temperature characteristics.

Since in reaction 1) the first step is a condensation of ammonia and $CO_2$ to carbamate, the carbamate formation under these conditions has interested us. No extensive studies have been made on it except that at the present time it seems unlikely that an enzymatic catalysis of this condensation occurs, at least in bacterial systems. The reaction between ammonia and $CO_2$ has a certain similarity to that between water and $CO_2$ which, as is well known from Roughton's work (9), is catalyzed by carbonic anhydrase. However, carbonic anhydrase does not catalyse a carbamate condensation (cf. Roughton, 9); we found that the addition of a rich source of carbonic anhydrase, as, for example, extracts of red blood cells, has no influence on the rate of the overall reaction.

It has been possible to show that two different enzymes catalyze reactions 1) and 2). The first indication of this was that heating at 70° C. for 3 minutes did not affect the carbamyl phosphate → citrulline enzyme; while, under the same conditions, the carbamyl phosphate → ATP enzyme is almost completely destroyed. Moreover, the enzymes can be separated by ammonium sulfate fractionation. As shown in Table II, the carbamyl phosphate → citrulline enzyme comes down first, leaving behind a reasonably concentrated solution of the carbamyl phosphate → ATP enzyme.

TABLE I.

*Rates of reactions catalyzed by* Streptococcus fæcalis *enzymes*

| Reaction | μ-moles of product/mg. protein/10 min. | | pH |
|---|---|---|---|
| | 0° C. | 30° C. | |
| ATP → citrulline . . . . . | 0.65 | 6.70 | 8.5 |
| CAP → citrulline . . . . . | 18.4 | 298 | 8.5 |
| CAP → ATP . . . . . . | 9.6 | 96 | 5.5 |

For the ATP → citrulline determination, each vessel contained in 1 ml. : 200 micromoles tris (hydroxymethyl) aminomethane buffer, pH 8.5; 10 micromoles of $MgCl_2$; 10 of ATP; 10 of ornithine and 50 of ammonium carbonate. For the CAP → ATP determination each vessel contained in 0.5 ml. : 100 micromoles acetate buffer, pH 5.5; 4 micromoles of $MgCl_2$; 4.8 of ADP; 4 of CAP. For the CAP → citrulline determination, each vessel contained in 0.5 ml. : 100 micromoles tris (hydroxymethyl) aminomethane buffer, pH 8.5; 5 micromoles of ornithine; 5 of CAP. A dialyzed preparation of the crude *Strep. fæcalis* extract was used for all experiments, and the concentration was so adjusted that two levels gave linear results and less than 20 % of the substrate was utilized.

TABLE II.

*Purification of* Streptococcus fæcalis *enzymes*

| | CAP → ATP | | CAP → Citrulline | |
|---|---|---|---|---|
| | Total units | Specific activity | Total units | Specific activity |
| A. Concentration : | | | | |
| 1. Original extract . | 77 000 | 61 | 305 000 | 240 |
| 2. 60-80 % sat. A. S. | 69 000 | 187 | 230 000 | 610 |
| B. Separation : | | | | |
| 3. Acid A. S. | | | | |
| *a*) 1.4-1.7 M . . . . . | 0 | 0 | 154 000 | 5 500 |
| *b*) 1.96-2.2 M . . . . . | 56 500 | 530 | 9 600 | 180 |

Enzyme activity was measured as described in legend of table I. All incubations were carried out at 30° C. for 10 minutes. One units is defined as that amount of enzyme which forms 1 micromole of product in 10 minutes at 30° C. Specific activity is expressed as units per mg. protein.

The carbamate-ATP reaction, as already described (1), requires magnesium; while the carbamyl transfer from carbamyl phosphate to ornithine does not. No further cofactor requirements have so far been found for either 1) or 2).

*Mammalian systems.* — Citrulline synthesis was postulated by Krebs as the first reaction in urea synthesis (10). This partial system has been studied extensively by Cohen and Grisolia (11). There is an important difference between the mammalian and the bacterial systems for, as Grisolia and Cohen found (12), the overall reaction in the mammalian system requires an additional cofactor, namely, an N-acylated glutamate such as acetyl or carbamyl glutamate. Recently, in a study of the phosphorolysis of citrulline, Krebs *et al.* have reported that, in addition to the glutamate derivatives, activity was also shown by carbamyl alanine (13).

We have confirmed the effect of carbamyl glutamate and similar compounds on the overall citrulline synthesis from ATP, ammonia and $CO_2$ using acetone powder extracts of beef liver. With these extracts an absolute requirement for such a compound was found, as shown in table III. In contrast, it was found that with the same enzyme a rapid reaction between carbamyl phosphate and ornithine occurs in the absence of such activators. It appears likely, from these experiments, that carbamyl glutamate and analoguous compounds function in the mammalian equivalent to reaction 1) of the bacterial system. The exact mechanism of this reaction in the mammalian enzyme system remains to be elucidated. Grisolia and Marshall have reported on the isolation of a compound X (14) which required for its formation the presence of a glutamate derivative in addition to the substrates of reaction 1). Compound X and carbamyl phosphate appear to behave similarly in some respects. Both react with hydroxylamine and hydrazine (*) (14) to give similar reactions in the Archibald color tests (15, 16); in addition, the half-lives of the two compounds at 37-38° C. are very nearly alike (17).

TABLE III.

*Citrulline synthesis with beef liver enzyme*

| Carbamyl source | Citrulline formed —Ac Glu | Citrulline formed +Ac Glu |
|---|---|---|
| | (μ-moles/ml.) | (μ-moles/ml.) |
| 1. ATP, $NH_4^+$, $HCO_3^-$ : | | |
| *a*) 3.1 mg. enzyme | 0 | 0.70 |
| *b*) 6.3 mg. enzyme | 0 | 1.45 |
| 2. CAP : | | |
| *a*) 0.03 mg. enzyme | 0.86 | 0.75 |
| *b*) 0.063 mg. enzyme | 1.65 | 1.62 |

All vessels were incubated at 37° C. for 30 minutes. Each vessel contained, in 1 ml. : 150 micromoles of tris (hydroxymethyl) aminomethane buffer, pH 7.9; 5 of ornithine; and, in addition, in experiment 1, 10 micromoles of ATP; 10 of $MgCl_2$; 10 of potassium bicarbonate and 10 of ammonium chloride; in experiment 2, 5 micromoles of CAP. Where indicated, 2.5 micromoles of acetyl glutamate were added.

From our incomplete studies we conclude that the first phase of citrulline synthesis is more complicated in the mammalian tissue than it is in the bacterial system. We hope that further studies will define this difference more clearly.

*Ureidosuccinate formation from carbamyl phosphate.* — A mentioned briefly, it was found that ureidosuccinate is formed by condensation of carbamyl phosphate and aspartic acid. The amount of ureidosuccinic acid formed was estimated indirectly by measuring the disappearance of aspartate (after removal of ammonia) by the ninhydrin procedure and by the determination of orthophosphate (table IV). The positive identification of ureidosuccinate was made by paper chromatography. As was the case with the citrulline system in extracts of *Streptococcus faecalis*, no activator is needed for the ureidosuccinate synthesis. However, in mammalian liver a stimulation of this reaction by glutamate derivatives has been reported (19, 20).

TABLE IV.

*Synthesis of ureidosuccinic acid by* Strep. fæcalis *extracts*

| Carbamyl source | Orthophosphate | Aspartic acid |
|---|---|---|
| | (μ moles/ml.) | (μ-moles/ml.) |
| A. ATP and $(NH_4)_2CO_3$ : | | |
| 1. Complete | 4.11 | 11.4 |
| 2. No $(NH_4)_2CO_3$ | 1.20 | 14.0 |
| 3. No $(NH_4)_2CO_3$ and aspartic acid | 1.14 | 2.0 |
| B. CAP : | | |
| 1. Complete-incubated | 6.02 | 9.4 |
| 2. Complete-unincubated | 1.77 | 14.0 |

All tubes were incubated at 30° C. for 30 minutes. In experiment A, the complete system contained in 1 ml. : 12 micromoles of L-aspartic acid; 5 of $MgCl_2$; 10 of ATP; 100 of ammonium carbonate; 200 of tris(hydroxymethyl)aminomethane buffer, pH 8.5 and 4 mg. enzyme. In experiment B, the complete system contained in 1 ml. : 12 micromoles of aspartic acid; 10 of CAP; 200 of tris(hydroxymethyl)aminomethane buffer, pH 8.5 and 4 mg. enzyme.

*Conclusion.* — It appears from these studies that the bacterial citrulline system can be clearly defined as composed of two separate enzymes catalyzing 1) the phosphorylation of carbamate by ATP, and 2) the transfer of the carbamyl group from carbamyl phosphate to ornithine. In the mammalian system as well carbamyl phosphate was found to serve as carbamyl donor to

(*) Carbamyl phosphate when heated at 100° C. with ammonia, hydroxylamine and hydrazine, yields urea, hydroxyurea and semicarbazide respectively. These products were identified by paper chromatography (18).

ornithine. However the initial synthesis of the carbamyl donor appears to be more complicated in the mammalian system and requires the presence of an activator such as carbamyl or acetyl glutamate.

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# The structure of the electron transport system of mitochondria


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(*Received* 9 *September* 1955)

Dr. Slater has provided us with an excellent review of the present status of the problem of oxidative phosphorylation, a problem to which he has made important contributions. The term 'oxidative phosphorylation' implies, as it was meant to imply, that esterification of inorganic phosphate is inextricably tied up with the process of terminal electron transport. Insofar as we may attempt to visualize the mechanism of oxidative phosphorylation, it can be done only in terms of what we know, or think we know about the electron transport system.

According to the classical picture of electron transport first developed by Keilin (1-7), and brought to its modern form by Slater (8-12) and Chance (13, 14) the passage of electrons from substrate to oxygen involves a series of at least 4 heme components or cytochromes which collaborate in shuttling electrons from the dehydrogenase system at the one end, to molecular oxygen at the other end of the chain. These heme components are assumed to be separate and separable entities which interact with one another by thermal or molecular collision.

At the present stage of our knowledge, how certain can we be that this classical picture of the electron transfer mechanism is a valid one ? If there are indeed 4 separate heme components which interact with one another serially, how can we account for the fact that to date only one has been isolated and shown to undergo the cyclical reduction and oxidation required by theory ? This failure might be a consequence of formidable technical difficulties, or it might be an indication that we have not visualized the electron transport system in the proper terms. There is of course only one way to find out, namely by way of the direct study of the electron transport system itself by the methods of isolation and purification. As you know various investigators, notably Wainio (15-19), Kiese (20), Stotz (21-24) and Ball (25,26) have made an excellent start in that direction. In the few minutes at my disposal I would like to summarize some of the observations made in our laboratory which bear on the structural pattern of the electron transport system, and then acquaint you with a few conclusions that we have reached so far.

The electron transport system of heart mitochondria can be isolated from heart mitochondria in the form of a single giant particle which we have called the electron transport particle or ETP (27). Some features of its composition are shown in table I. It contains hemes

TABLE I

*Composition of preparations of ETP*

| | Average value (μ-moles or μ-atoms/g. enzyme) |
|---|---|
| Non-heme Fe | 18.3 |
| Cu | 2.4 |
| Flavin | 0.3 |
| Heme | 2.5 |
| Lipid | 35 % |

of two types which for convenience we shall refer to as red and green hemes, and in addition, flavin, non-heme iron, copper and lipide. Per mole of flavin ETP contains in round numbers 8 moles of heme, 61 atoms of non-heme iron and 8 atoms of copper.

The enzymatic characteristics of ETP can be summarized in the following manner. First, it catalyzes the oxidation of both succinate and DPNH by oxygen without any additions, and the rate of oxidation is not stimulated by addition of cytochrome *c*. Second, all the hemes of ETP are immediately and completely reduced on addition of either succinate or DPNH. Third, cytochrome *c* does not appear to be a constituent of the particle. Fourth, the reduction of added cytochrome *c* by succinate or DPNH in presence of ETP proceeds very slowly, if at all. The same is true of the oxidation of reduced cytochrome *c* by oxygen.

The problem of deducing the arrangement of the various structural elements of ETP is not unlike that of deducing the structural formula of a complex alkaloid. By controlled degradation of ETP, smaller component, fragments can be obtained (some with catalytic activity) and others without activity depending upon the original location of these fragments in the electron transport chain. Also, in degrading ETP, succinic or DPNH dehydrogenase can be detached as such, or in combination with other elements such as metal, heme, lipide etc. Each of these increasingly more complex forms shows dehydrogenase activity, but the electron acceptor pattern differs from one form to another, since this pattern depends upon the nature of the groups combined with the dehydrogenase.

Let us consider the various forms of succinic dehydrogenase which can be obtained by degradation of ETP. When ETP is exposed to dilute amyl alcohol, under special conditions, the particle is fragmented into a red particle containing only red hemes and a green particle containing only green heme. This red particulate fraction we have named succinic dehydrogenase complex or SDC (28). This has two forms which for convenience we refer to as the high lipide form and the low lipide form. The high lipide form has relatively high succinic activity while the low lipide form is less active in this respect. Now, by exposure to crude trypsin, SDC can be converted to a soluble form whose heme content can be reduced stepwise by further exposure to tryptic digestion until all the heme has been removed. Finally, a non-heme containing flavoprotein with succinic dehydrogenase activity is formed (29). There are, in fact, other and more efficient ways of preparing the flavoprotein from SDC but we shall not go into that in this brief talk.

A rough guide to the composition of these different forms of succinic dehydrogenase is given in table II. You will note that flavin is present in all active fractions, including the simplest unit with succinic dehydrogenase activity. As the units become progressively simpler, lipide, heme, and non-heme iron are removed, until finally a simple ferroflavoprotein is left as the least common denominator of enzymatic action.

The electron acceptors for the different species of succinic dehydrogenase are shown in table III. In the original ETP, oxygen is the acceptor of choice; cytochrome *c* is virtually inactive. Immediately after amyl alcohol treatment, oxygen can no longer serve as electron acceptor but cytochrome *c* does very well. When the heme moiety is removed, or reduced in size, then cytochrome *c* falls out as electron acceptor and ferricyanide or phenazine are the acceptors of choice. According to Singer and Kearney (30), a simpler flavoprotein than the one we have isolated can be prepared which works well with phenazine but not with ferricyanide.

A similar series, though not quite as extensive, has been elaborated for DPNH dehydrogenase (31, 32), but we will not have time to consider the details of the DPNH series. The principle and general nature of the results are much the same as for the succinic dehydro-

TABLE III

*Electron acceptors for different species of succinic dehydrogenase*

| | $O_2$ | Cyt. *c* | Ferricyanide | Phenazine (*) | Indophenol |
|---|---|---|---|---|---|
| ETP | + | 0 | + | + | + |
| SDC | 0 | + | + | + | + |
| Flavoprotein | 0 | 0 | + | + | + |

(*) Phenazine methosulfate.

TABLE II

*Composition of different species of succinic dehydrogenase*

| | Flavin | Total heme | Red heme | Green heme | Non-heme Fe | Cu | Lipide |
|---|---|---|---|---|---|---|---|
| I. ETP (*) | 1 | 8 | + | + | 61 | + | + |
| II. SDC (**) — h. l. (high lipide) | 1 | 4 | + | 0 | 16 | 0 | + |
| III. SDC (**) — l. l. (low lipide) | 1 | 4 | + | 0 | 16 | 0 | + |
| IV. Soluble flavohemoprotein | 1 | 4 | + | 0 | + | 0 | 0 |
| V. Soluble flavohemoprotein | 1 | 1 | + | 0 | + | 0 | 0 |
| VI. Soluble flavoprotein | 1 | 0 | 0 | 0 | 1 | 0 | 0 |

(*) ETP = Electron tranport particle.
(**) SDC = Succinic dehydrogenase complex.

genase series. Sufficient to say that we have high hopes that by extension and amplification of these degradative procedures we will be able to provide a precise formulation of the arrangement of heme, flavin, non-heme iron, copper and lipide in ETP. More important, we may thereby gain some insight into the molecular structure of the electron transport system without which we can only grope in the dark for an understanding of oxidative phosphorylation.

We have examined ETP for the presence of cytochrome *c* using a variety of methods for detection. Thus far we have had no success in demonstrating the presence of more than an insignificant trace of cytochrome *c*.

We should stress that the absence of cytochrome *c* as part of the electron transfer sequence in ETP does not necessarily mean that cytochrome *c* plays no role in the overall process. My colleagues, Drs. Jacobs and Sanadi (33), have indeed established that cytochrome *c* is essential for the oxidation of both succinate and DPNH in a liver mitochondrial system which is carrying on oxidative phosphorylation. There is a real dilemma based on the requirement of cytochrome *c* for mitochondrial oxidation, and its absence from the isolated ETP, which has yet to be resolved.

This symposium would appear to be an appropriate occasion to provide some idea of our present concept of the structure of the electron transport system based on these stepwise degradation studies. We are thinking in terms of the electron transport system as a quasi-conducting continum in which electrons originating in DPNH and succinate are transferred ultimately to molecular oxygen. The various oxidation-reduction groups of this semi-conductor system are linked to one another by bonds which permit resonance interaction throughout the entire structure. In a limited sense, the movement of electrons is like that of conduction of electrons through a metal. However the analogy is not complete, since the identity of the electron is indeterminate in a metal, while to a first approximation the same electron which left the substrate eventually turns up in molecular oxygen. Secondly, there are restraints to the unimpeded flow of electrons in ETP which do not apply to a metal. Perhaps most significant, is the fact that classical kinetics cannot describe the mechanism by which electrons move through the system. The notion of concentration of reactants, of collisions between elements in the chain, or of any other feature of the collision mechanism is, in our opinion, inapplicable to the ETP. Classical kinetics is applicable only at the beginning of the process in the interaction of substrate and dehydrogenases, and at the end in the interaction between cytochrome $a + a_3$ and molecular oxygen. In the study of metalloflavoproteins (34) we are faced with much the same dilemma. It is impossible to apply classical kinetics to the interaction of the metal and flavin in a metalloflavoprotein. In a resonating system the movement of the $\pi$-electrons from one oxidation-reduction element to another does not depend upon intramolecular collisions, and hence the concept of mass action is inapplicable to this situation.

There is some circumstantial evidence which leads us to speculate that the large number of non-heme irons is concerned, at least in part, with providing the structural links between the different oxidation-reduction elements of the system. There is insufficient evidence at present to do more than speculate on the molecular arrangement and structure of the quasi-conductor system. But any satisfactory theory will have to explain the presence, not only of the considerable concentrations of iron and copper, but also of lipide. How the hemes can be linked to one another through iron atoms is still a real puzzler. Finally, how copper collaborates with the green hemes in catalyzing the reaction with molecular oxygen is one of the challenges for future experiment.

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## Studies on the biosynthesis of citrulline and carbamyl aspartate

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(*Received* 15 *August* 1955)

The chemical synthesis and activity of carbamyl phosphate in citrulline and carbamyl aspartate biosynthesis in *Streptococcus faecalis* R and liver has led Dr. Jones and her coworkers to consider this compound a naturally occurring intermediate in biological systems (1, 2). Dr. Grisolia in my laboratory isolated a partially purified compound as the cyclohexylammonium salt which fulfilled the requirements of an intermediate in citrulline biosynthesis (3). This derivative contained one mole each of $CO_2$, $NH_3$, and organically bound phosphate making up a carbamyl phosphate group. A mole of carbamyl glutamate was considered also to be present but it is now known that this was an artifact due to a reaction between carbamyl phosphate and cyclohexylamine to produce a chromogenic compound (4). With the development of techniques in our laboratory for the isolation and purification of this intermediate from animal systems, it was possible to study the role of this intermediate in bacterial systems. Studies initiated last fall by Drs. Knivett and Marshall (5) using *Streptococcus faecalis* (ST) (NTC N° 6782), soon revealed that the animal and bacterial intermediates were interchangeable and further that no glutamyl derivative (GD) was necessary for the synthesis of the intermediate in the case of the bacterial system. These studies clearly indicated that the intermediate formed in the case of the bacterial system was carbamyl phosphate.

The identity of the animal and bacterial intermediates and the identity of synthetic carbamyl phosphate with the intermediates isolated from liver systems remained to be established. It is now clear from recent studies (4) in our laboratory that the intermediate isolated from liver systems in our earlier studies on citrulline biosynthesis is indistinguishable from carbamyl phosphate. Further, as reported by Dr. Jones *et al.* (2), carbamyl phosphate is also involved in the synthesis of carbamyl aspartate, a system which has been studied in some detail by Dr. Lowenstein (6) and by Dr. P. Reichard (7). The earlier findings by Dr. Grisolia and myself (8, 9, 10, 11) that a glutamyl derivative is required for the synthesis of the intermediate in animal systems has been confirmed by other investigators during the past few years (12, 13), and most recently by Dr. Jones *et al.* (1).

The absence of a requirement of a glutamyl derivative for citrulline synthesis by *S. faecalis* is shown in table I. Since this microorganism contains an active acetokinase, an enhancement of citrulline synthesis is readily demonstrated by the addition of acetyl phosphate (figure 1).

TABLE I

*The absence of a requirement for a glutamate derivative for citrulline biosynthesis by extracts of* S. faecalis

| Experiment | Remarks | Intermediate formed (μ-moles) | Citrulline formed (μ-moles) |
|---|---|---|---|
| 1 | Complete system | 3.92 | 10.20 |
| 2 | AG(*) excluded | 3.92 | 10.20 |
| 3 | Muscle excluded | 0.15 | 3.00 |
| 4 | Muscle and AG excluded | 0.15 | 2.95 |
| 5 | 3-PGA (**) excluded | 0.14 | 3.39 |
| 6 | 3-PGA and AG excluded | 0.15 | 3.30 |

(*) Acetyl-L-glutamate.
(**) D-3-phosphoglycerate.

Final concentration of substrates in the incubation mixture were as follows : ATP, $3 \times 10^{-2}$ M ; $MgSO_4$, $2 \times 10^{-2}$ M ; $NaHCO_3$, $1 \times 10^{-1}$ M ; $NH_4Cl$, $3 \times 10^{-2}$ M ; 3-PGA, $2 \times 10^{-2}$ M ; AG, $4 \times 10^{-3}$ M ; and when citrulline synthesis was measured, ornithine $5 \times 10^{-3}$ M. Each tube contained 0.5 mg. *S. faecalis* extract (ST5D) and 3 mg. of muscle preparation. Final volume, 2.5 ml. Incubation time, 20 minutes at 38° C. The intermediate was measured as citrulline by converting aliquots of the deproteinized supernates with Enzyme II plus ornithine.

The scheme which we have previously proposed for synthesis of the intermediate and citrulline (represented by reactions *1* and *2a* of figure 2) requires that the glutamyl derivative act catalytically in the system leading to citrulline synthesis. However, if the reaction were stopped at the GD-intermediate stage, the amount of intermediate formed should bear a stoichiometric relationship to the amount of glutamyl derivative added to the system. Recently published experiments (4) have revealed that acetyl glutamate (and carbamyl glutamate) acts catalytically in the synthesis of the intermediate. Further, the intermediates formed in the presence of acetyl glutamate and carbamyl glutamate and isolated as cyclohexylammonium salts (5) did not function catalytically but rather stoichiometrically in a system which supported citrulline synthesis with catalytic amounts of added acetyl glutamate or carbamyl glutamate. These experiments, in addition to others, clearly exclude the presence of a glutamyl derivative in the intermediate isolated.

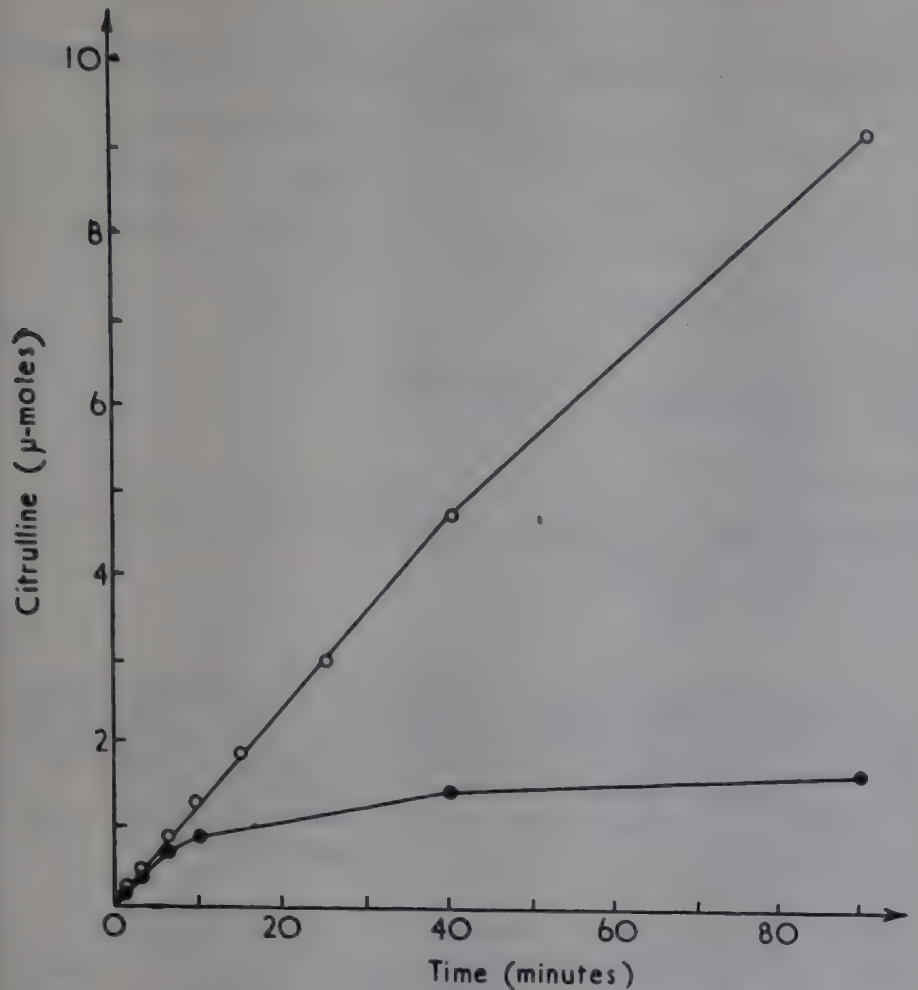


FIG. 1. — Acetyl phosphate as a source of energy rich phosphate for the synthesis of citrulline by extracts of *S. faecalis*. Curve B represents citrulline synthesis in the presence of acetyl phosphate. Curve A represents citrulline synthesis in the absence of acetyl phosphate. Final concentrations of substrates in all experiments were as follows: ATP $1 \times 10^{-3}$ M; $NH_4HCO_3$, $3 \times 10^{-1}$ M; MgCl, $2 \times 10^{-3}$ M; ornithine, $1 \times 10^{-2}$ M. In the experiments of curve B, acetyl phosphate was added to $1 \times 10^{-2}$ M final concentration. Each tube contained 0.06 mg. of *S. faecalis* extract. Final volume, 1.0 ml. Incubation at 38° C.

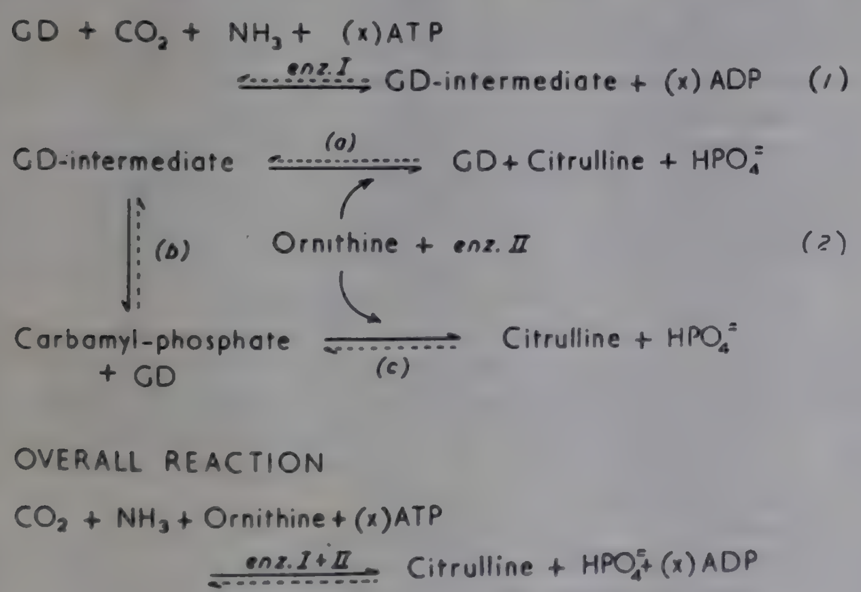


FIG. 2. — G D = glutamyl derivative.

The fact that the animal system requires a glutamyl derivative for intermediate and citrulline synthesis indicates that a glutamyl intermediate of some kind is formed, either on the enzyme or as a free compound. The possibility exists that such an intermediate can react with ornithine to form citrulline in accordance with reaction *2a* (figure 2). On the other hand, it appears more likely that reactions *2b* and *2c* represent the main, if not the only pathway. The fact that synthetic carbamyl phosphate behaves identically with the intermediate synthesized enzymatically and the fact that the glutamyl derivative acts catalytically in the synthesis of the intermediate support the pathway represented by reactions *2b* and *2c*. However, evidence for the formation of an acetyl-glutamyl carbamyl phosphate intermediate as well as carbamyl phosphate in a liver system has recently been obtained (7).

Previous studies from this laboratory (3, 5) and more recently from Dr. Grisolia's laboratory (14) have indicated that the decomposition of the cyclohexylammonium salt of the isolated intermediate decomposed at pH 5 with the liberation of inorganic phosphate, $CO_2$ and $NH_3$. While the liberation of $NH_3$ from the cyclohexylammonium salts of isolated intermediates is slower than from synthetic lithium carbamyl phosphate, the sodium salts yield $NH_3$ at the same rate (15) (figure 3).

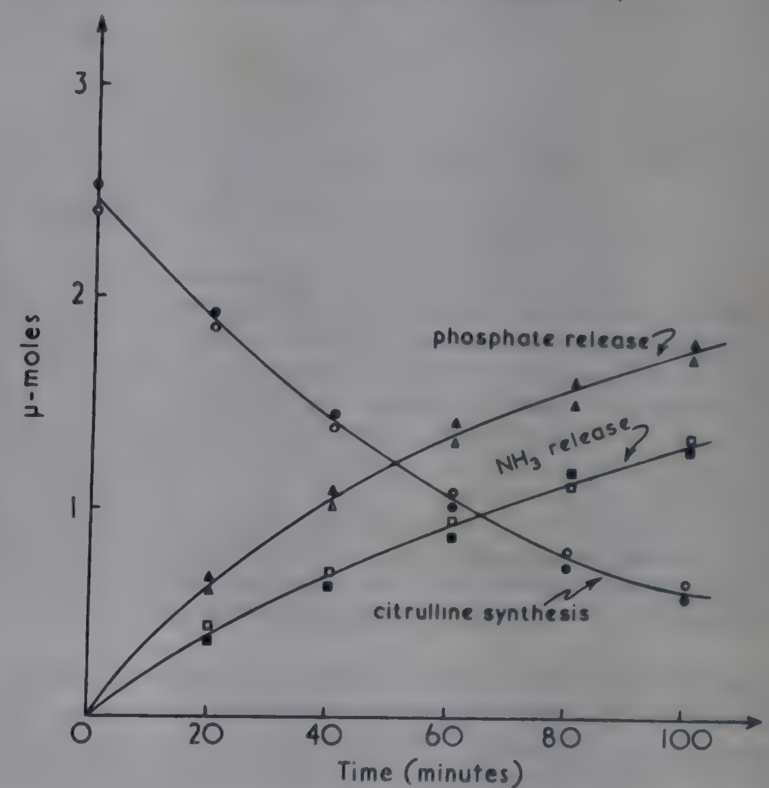


FIG. 3. — Comparison of the decomposition of the sodium salts of carbamyl phosphate and acetyl glutamate intermediate. The cyclohexyl-ammonium salt of AGI, and the lithium salt of CAP were converted to the sodium salts by passing through sodium Dowex-50. Recovery was essentially 100 %. Decomposition was allowed to proceed at 38° C. in 0.08 M acetate buffer, pH 5.0. At the time intervals noted ammonia was determined by direct nesslerization; phosphate by the method of Lowry and Lopez; and the intermediates remaining by conversion to citrulline using enzyme II plus ornithine. It has been found that essentially no ammonia release occurs with nesslers reagent. Similarly, only slow release of phosphate occurs with the Lowry and Lopez reagent. Open symbols = sodium salt of acetyl glutamate intermediate. Solid symbols = sodium salt of carbamyl phosphate.

From the data presented, it is clear that the compound isolated in our laboratory from liver systems several years ago was in fact carbamyl phosphate, and thus provides clear proof for the biological intermediate role of carbamyl phosphate.

Published studies on the synthesis of carbamyl aspart-

ate (13, 16, 17, 18) have established that the same intermediate is involved in the biosynthesis of both citrulline and carbamyl aspartate. Data supporting the identity of the intermediates in carbamyl aspartate and citrulline synthesis (6) are shown in figure 4. The data in figure 4

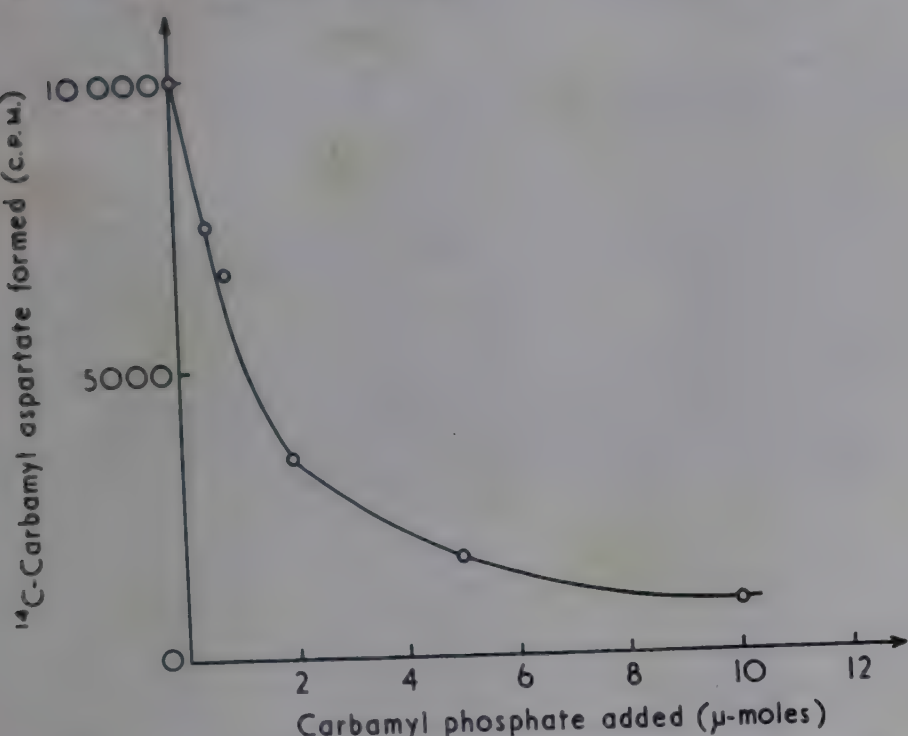


Fig. 4. — The incorporation of $^{14}$C-labeled animal intermediate into carbamyl aspartate in the presence of increasing amounts of synthetic carbamyl phosphate. Each tube contained : aspartate, 20 µ-moles ; diethanolamine-HCl buffer, pH 9.2, 200 µ-moles ; $^{14}$C-labeled animal intermediate, 1.1 µ-moles ($5.95 \times 10^4$ c. p. m./µ-mole) ; dialysed, lyophilized low speed rat liver supernatant, 10 mg. protein ; and carbamylphosphate as indicated. Final volume 2.0 ml., 38° C., 15 minutes after addition of aspartate. CA formed was assayed after separation by ion-exchange chromatography. Circles : experimental points ; solid line : curve calculated on the assumption that the animal intermediate and carbamylphosphate are identical.

shows that if unlabeled carbamyl phosphate is added to a complete system containing $^{14}$C-labeled intermediate, the carbamyl aspartate formed has a decreasing specific activity as the concentration of added carbamyl phosphate is increased. The predicted dilution curve by the non-radioactive intermediate falls along the observed points, thus supporting the identity of the two compounds.

While the synthesis of citrulline in animal tissues is limited to liver, the synthesis of carbamyl aspartate takes place in many tissues, and the transcarbamylase activity is actually higher in intestinal mucosa and testes than in liver (6). An enzyme activity comparison of carbamyl aspartate synthesis in different tissues is shown in table II. The carbamylation of amino acids was found to occur only in the case of ornithine, aspartate, asparagine, *iso*asparagine, and lysine (6). Carbamyl aspartic acid has recently been isolated from rat liver (19).

The nature of the 'physiological' glutamyl derivative active in carbamyl phosphate synthesis is still under investigation. An active fraction has recently been isolated from rat liver (15).

Recent kinetic and inhibition studies by Knivett (20) reveal that carbamyl phosphate inhibits the arsenolysis of citrulline by *S. faecalis* preparations and is not itself

Table II

*Distribution of carbamyl phosphate-aspartate transcarbamylase in rat tissues*

| Tissue | Relative activity |
|---|---|
| Intestinal mucosa . . . . | 100 |
| Testes . . . . . . . . . . | 55 |
| Liver . . . . . . . . . . | 51 |
| Kidney . . . . . . . . . | 35 |
| Lung . . . . . . . . . . | 29 |
| Spleen . . . . . . . . . | 21 |
| Brain . . . . . . . . . . | 20 |
| Pancreas . . . . . . . . | 19 |
| Muscle (skeletal) . . . . . | 8 |
| Muscle (heart) . . . . . . | 1 (?) |
| Blood (whole) . . . . . . | 1 (?) |

Each tube of the test system contained : C$^{14}$-carbamylphosphate, 2.0 µ-moles (22 500 c.p.m.) ; aspartate, 10 µ-moles ; DEA buffer, pH 9.2, 100 µmoles ; and high speed supernates as shown, 3 to 10 mg. protein. Blanks contained no aspartate.

Final volume 1.0 ml., 38° C., 15 minutes after addition of carbamylphosphate. Assays performed in duplicate.

readily decomposed by arsenolysis. If ornithine or ADP plus $Mg^{++}$ are added the arsenolysis of citrulline is rapidly initiated owing to the removal of carbamyl phosphate by citrulline formation in the former case (transcarbamylation) and ATP, $CO_2$ plus $NH_3$ formation in the latter case (carbamate kinase).

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# Energetic coupling and the regulation of metabolic rates

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(*Received* 13 *June* 1955)

One of the characteristics of more highly evolved organisms is their facility for varying the intensity of their metabolism according to the needs of the moment. The energy metabolism of man can increase during work by a factor of tenfold that during rest. In contrast, most microorganisms provided with an abundance of nutrients can only metabolize at a rapid rate, assimilate nutrients and continue to multiply as long as the nutrients last. Yet facultative microorganisms possess some ability to regulate their metabolic processes, for, when oxygen is available they employ the metabolically efficient oxidation system in preference to glycolysis or fermentation.

The regulatory processes are more complicated in the more highly evolved forms of life and are most elaborate in the mammals which show a remarkable ability to cope with environmental adversity.

Among the organs of the body which have been studied, all seem to metabolize more rapidly when stimulated to perform their normal functions. Loewy (1) has tabulated the degree of enhancement of energy metabolism during activity as follows : kidney, 4 to 5 fold ; salivary gland, 3 fold ; pancreas, 4 fold ; heart, 10 fold. In nerve this enhancement of metabolism as a result of stimulation is even greater (fig. 1). Feng and Hill (2) found that the increase in steady-state rate of heat production by nerve is a function of the frequency of the impulses, and Brink, Bronk, Carlson and Connelly (3) find that this holds also for the increase in steady state rate of oxygen consumption (fig. 1).

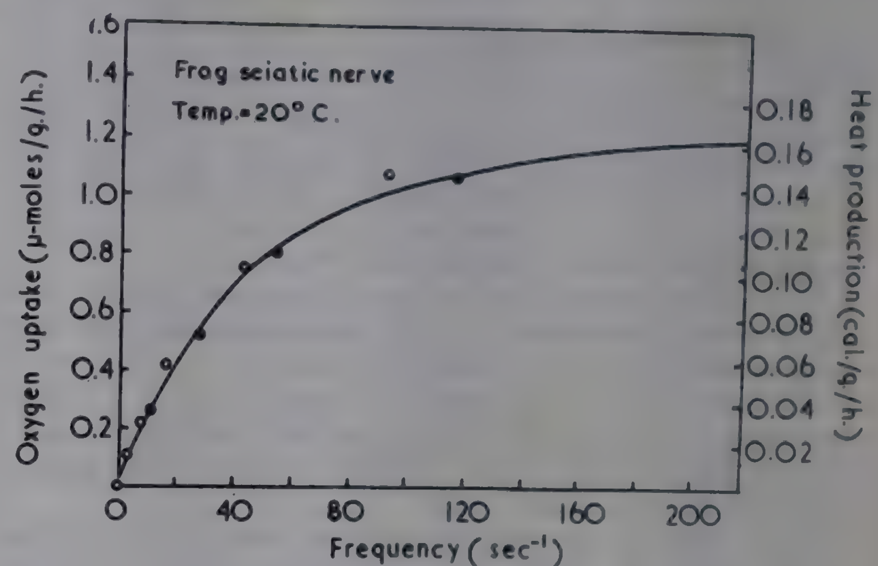



Fig. 1. — Oxygen consumption and heat production of frog nerve as a function of the frequency of the impulses. From Brink, Bronk, Carlson and Connelly (2).
○ Increase in steady-state rate of oxygen uptake on stimulation (*Rana pipiens*).
● Increase in steady-state rate of heat production on stimulation (*Rana esculata*). From Feng and Hill (1933).

TABLE I

*Oxygen consumption of man and of his working leg muscles as affected by work load*

| | Work performed (kilogram-meters/minute) | | | | | |
|---|---|---|---|---|---|---|
| | Rest | 360 | 540 | 720 | 900 | 1080 |
| | ml./min. | ml./min. | ml./min. | ml./min. | ml./min. | ml./min. |
| Oxygen consumption of : the subject . . . . . . . | 255 | 1120 | 1440 | 1730 | 2075 | 2550 |
| the working muscle (per kg. basis) . . . . . . | 1.7 | 32.3 | 63.7 | 60.5 | 89.5 | 112 |

From Asmussen, Christensen and Nielsen, *Skand. Arch. Physiol.*, 1939, **82**, 212.

The mammalian striated muscle is a good example for discussing regulatory processes for it has been examined by a great variety of experimental sciences. The rate of combustion in muscle is known to vary tremendously depending upon its work output. During rest the oxygen consumption of muscle is low. Blood circulation is at a minimum, for, as was shown by Krogh (4), only one capillary of every hundred may be open and carrying blood. Not only is the blood supply restricted, the arteriovenous differences in oxygen saturation are much smaller during rest than during work (5). From these facts we may conclude that the decreased respiration of resting muscle results not from a restriction of oxygen supply, but rather that the decreased blood supply is, in some manner, a response to the decreased demand for oxygen.

Quantitative data are required to comprehend the magnitude and dramatic nature of the regulation of metabolism in muscle (table I). During rest the oxygen consumption of human muscle is approximately : 1.7 ml./min./kg. (6). A work load of 360 kg.-meters/min. may quadruple the total energy metabolism of the human body while increasing the oxygen consumption of the working leg muscles to 32.3 ml./min./kg., an increment of nearly 20 fold. Increasing the work load to 1080 kg.-meters/min. raises the oxygen consumption of the muscle to 112 ml./min./kg. It is estimated (6) that with maximum exertion the oxygen consumption of the muscle would increase 100 fold.

It is the purpose of this paper to consider some of the biochemical phenomena which are responsible for this type of control over oxidative processes. The question to be answered is, why does the respiration of a tissue abundantly supplied with oxygen and oxidizable substrates proceed only rapidly enough to supply the energy required at any given time?

There are, of course, an infinite variety of factors which influence rates of metabolism under varying conditions. A list of only a few which are operative in the living cell should certainly include :

(*i*) the concentration of enzymes,

(*ii*) the percentage of enzyme in its active form (we must now extend the concept of the zymogens to the enzymes of intracellular metabolism ; the phosphorylase $a \rightleftarrows b$ interconversion is one such example),

(*iii*) the spacial distribution of enzymes within the cell,

(*iv*) reactant concentrations and the oxidation-reduction potentials of the interacting components,

(*v*) coenzyme concentration,

(*vi*) activator (*e.g.*, metal ions) concentration,

(*vii*) the pH at the intracellular site of the reaction may influence the rate of reaction by its effect on the substrate as well as on the enzyme,

(*viii*) other environmental factors such as ionic strength, adsorption of the enzyme on (or occlusion of the enzyme by) a lipid phase, etc.,

(*ix*) temperature (and this may vary significantly, even in homeothermic animals, as a result of heavy work, adverse environmental temperature, an excess or deficiency of thyroid hormones, or sympathomimetic amines),

(*x*) rate of product removal,

(*xi*) the presence of natural inhibitors.

Many of these factors have been investigated in relatively simple systems and some are now being studied in more complex particulate fractions of cells or even in intact cells. Probably all of these factors affect the ' energetic coupling ' phenomena but those which have a particularly obvious role in the dynamic regulation of cellular oxidations, and which we shall discuss specifically, are the reactant concentration, the rate of product removal, and, probably, the concentration of certain activator ions.

A variety of experiments indicate that the reactants whose concentration may limit rates of oxidation are inorganic phosphate and any of the variety of phosphate acceptors. It seems likely that one of the 'products' which accumulate in resting muscle when recovery is complete is a phosphorylated form of an oxidation-reduction catalyst. Divalent cations are required for the

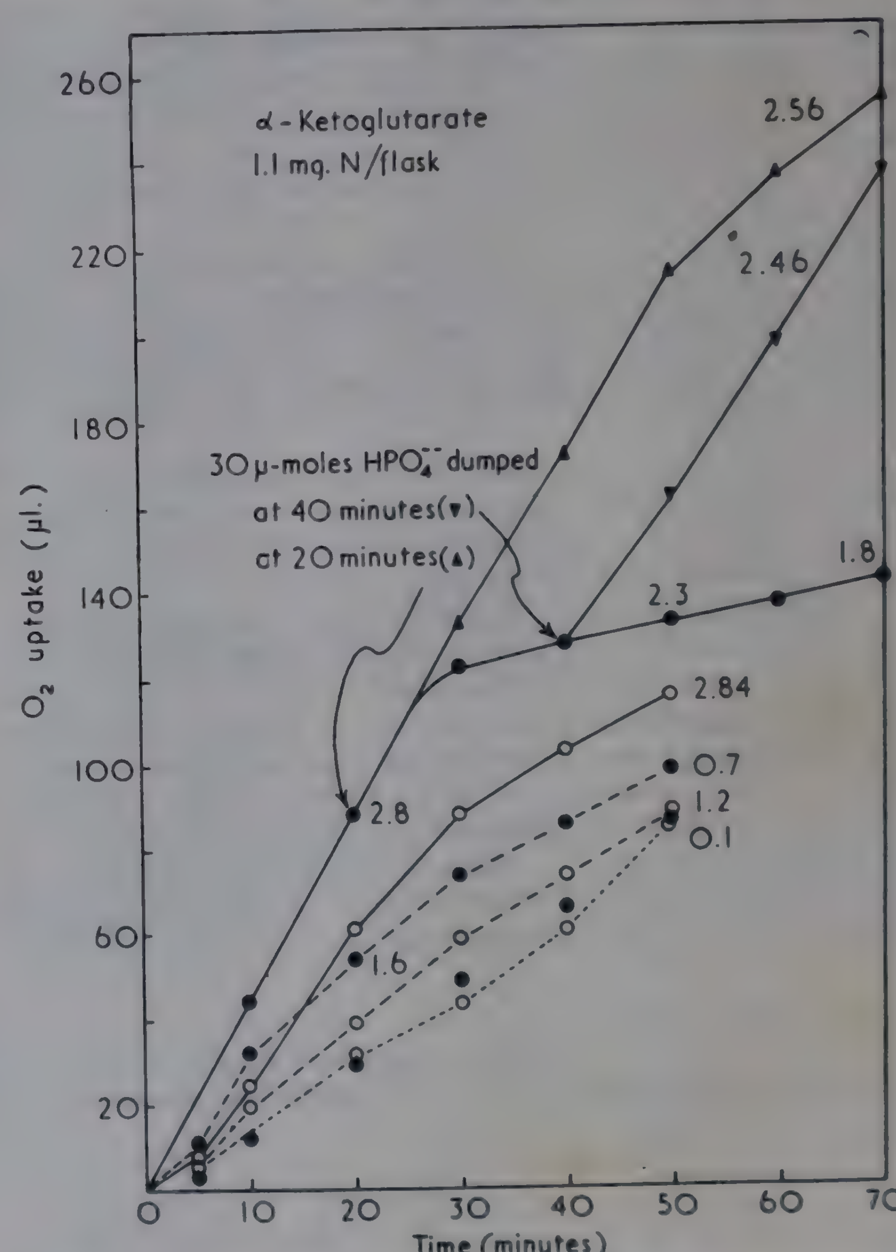



**Fig. 2.** — Effect of inorganic phosphate on rates of oxidation by rat liver mitochondria. Temperature : 30° C. The reaction mixture contained $2.2 \times 10^{-6}$ μ-moles cytochrome *c*, 3.6 μ-moles ATP, 30 μ-moles potassium phosphate pH 7.4, 15 μ-moles $MgSO_4$, 21 μ-moles α-ketoglutarate and mitochondria in 0.25 M sucrose to make 2 ml. final volume. The numbers in the figure refer to P : O ratios from flasks removed at various time intervals. Solid line = hexokinase and glucose added as phosphate acceptor. Dash line = creatine transphosphorylase and creatine added as phosphate acceptor. Dotted line = no acceptor. **O** = 0.01 M fluoride added.

coupling of phosphate fixation with respiration and for the transfer of the high-energy phosphate ($\sim$P). Potassium ions are required for at least one of these transphosphorylations.

### *Role of inorganic phosphate and phosphate acceptor in controlling rates of metabolism*

The possibility that $\sim$P acceptor concentration could exert a regulatory effect on metabolism was proposed by Engelhardt (7) and Lennerstrand (8). In his early studies of oxidative phosphorylation, Belitzer (9) found that additions of the $\sim$P acceptor, creatine, accelerated respiration and recognized the possibility that acceptor concentration and the concentration of inorganic phosphate ($P_i$) might act in the control of cellular respiration. Later Johnson (10) and Lynen (11) stated the problem in formal argument, particularly as it applies to the phenomena commonly called the Pasteur effect. Their analysis of the Pasteur effect is so completely adequate that this phenomenon needs no further discussion here.

Despite the apparent validity of the theory that $P_i$ and $\sim$P acceptor can regulate metabolism, the experimental results with animal tissues did not conform to predictions. The largest effect of a phosphate acceptor on respiration of surviving tissues was the 50 to 100 % stimulation observed by Belitzer (9) (scarcely enough to account for the control observed in intact tissues). Cross, Taggart, Covo and Green (12) obtained good yields of phosphate esterification coupled with oxidation by the particulate cyclophorase preparation, but respiration proceeded equally well without acceptor which indicated that phosphorylation might not be a compulsory concomitance of respiration. The problem remained to demonstrate the degree of control which $P_i$ and $\sim$P acceptor concentrations might exert in intact cells. While the problem is difficult to examine in whole cells, new developments in cell fractionation technique (Hogeboom) permit it to be studied with the oxidizing particles of the cell.

The combustion of metabolites in the cell takes place largely in the mitochondrial particles. When these particles are isolated from other cell components by homogenization and fractional centrifugation in isotonic solutions of nonelectrolytes, their respiration is so securely geared with the phosphate fixing process that oxygen consumption is almost entirely dependent upon added inorganic phosphate and phosphate acceptor (fig. 2 and 3).

In the presence of a $\sim$P acceptor-system such as glucose plus hexokinase, mitochondria respire at a steady rate until most of the inorganic phosphate is fixed. A new, slower, rate of respiration then occurs. Analysis of the flask contents at this point shows that the $P_i$

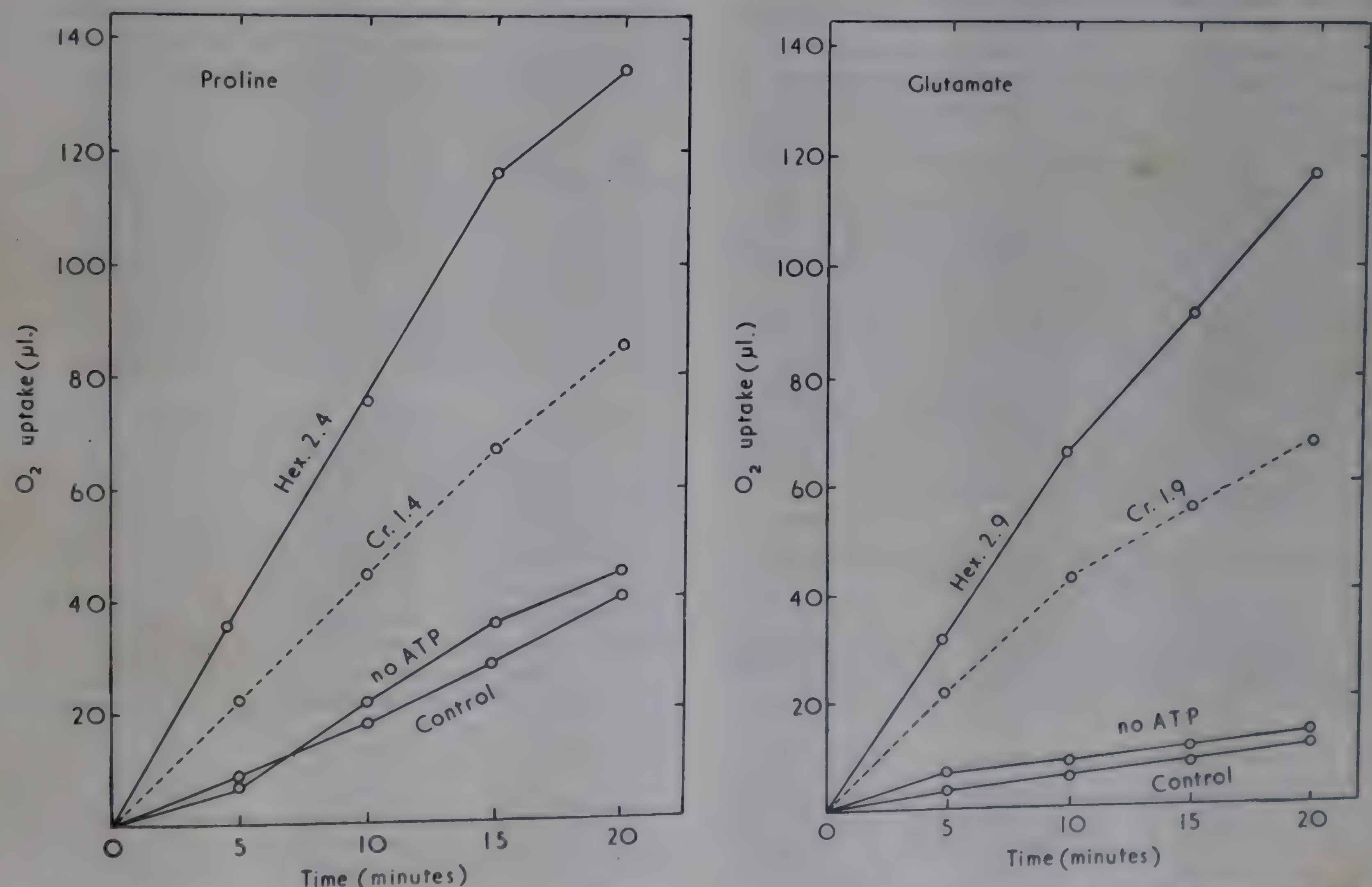



Fig. 3. — Effect of phosphate acceptors on oxidation by rat liver mitochondria. Conditions as in figure 2 except for the substrate added. Hex. = hexokinase and glucose as phosphate acceptor. Cr. = creatine transphosphorylase (65) and creatine as phosphate acceptor. The numbers indicate the P:O ratio for the 20 minutes period.

content has been reduced to about $2 \times 10^{-4}$ M. It can easily be demonstrated that it is the concentration of $P_i$ which is limiting respiration in this case, for if $P_i$ is added before the rate of oxygen consumption falls off, the initial rate is maintained, and if $P_i$ is added after the rate of oxidation has decreased, the original rate is restored. From this it is apparent that the $P_i$ released from ATP and phosphocreatine during muscle contraction would enhance the rate of muscle respiration and that once contraction has stopped, respiration could continue at the enhanced rate only until the $P_i$ had been brought to a low concentration again.

The fact that phosphate acceptor concentration can control rates of oxidation by mitochondrial preparations has been demonstrated in many laboratories (13-18) and with mitochondria from a variety of organs. Results similar to those shown in figure 3 have been obtained with citrate, α-ketoglutarate, succinate, malate, pyruvate, β-hydroxybutyrate and caprylate although the degree of stimulation by ~P acceptor varies somewhat depending upon the nature of the substrate. For example the oxidation of succinate or caprylate is seldom increased more than fourfold by addition of an acceptor system. Glutamate oxidation is stimulated five to 15 fold while the oxidation of β-hydroxybutyrate is frequently entirely dependent upon the presence of ~P acceptor. These differences have implications which will be discussed in connection with sites of phosphorylation.

There is, as yet, no definite information as to whether the $P_i$ concentration or ~P acceptor concentration is the more important factor in blocking respiration in resting muscle or other tissues. Resting muscle has been reported (24, 25) to contain 7 mM $P_i$. However, this is higher than the concentration (2 mM) required for half maximum respiration (18) and far in excess of the concentration to which mitochondria can bring $P_i$ in the presence of hexokinase and glucose as ~P acceptor (13). Dubuisson (26) assumes that the free creatine in resting muscle is equivalent to the $P_i$. It is a difficult technical problem to measure the $P_i$ content of resting tissues, for all procedures which are used in deproteinizing prior to analysis are apt to cause hydrolysis of labile esters. For a better understanding of the regulation of cellular metabolism we shall need more information about the concentration in tissues of $P_i$, phosphocreatine, creatine, and each of the nucleotides.

### *Effectiveness of individual sites of phosphorylation in respiratory control*

The sequence of electron transport in mitochondria in which the phosphorylation reactions are closely coupled with respiration appears to be as follows : (19)

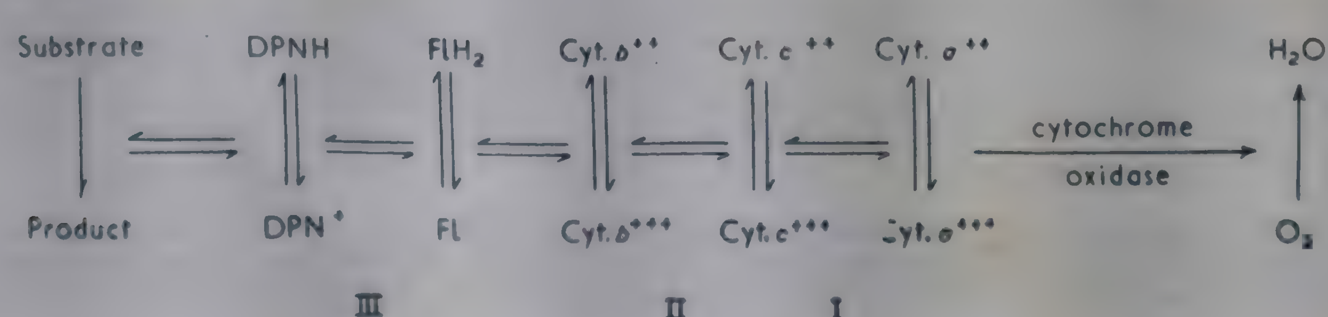
During the oxidation of substrates whose dehydrogenases are linked with pyridine nucleotides, nearly three moles of phosphate are fixed per atom of oxygen consumed (20). One of these phosphorylation sites (I) is located between reduced cytochrome *c* and oxygen (21-23). Chance and Williams have recently placed this phosphorylation at the step where cytochrome $c^{++}$ is oxidized by cytochrome $a^{+++}$ (19). The recurrence of a phosphorylation in this region of single electron transport poses the important question of how the oxidation of 2 molecules of cytochrome $c^{++}$ is coupled with uptake of a single molecule of $P_i$. The other two phosphorylations appear to occur prior to the point where ferricyanide interrupts the electrons from DPNH for $P : 2\ Fe(CN)_6^{---}$ ratios greater than 1 can be obtained with D-β-hydroxybutyrate as the substrate (27). Chance and Williams have placed these phosphorylations at the steps where cytochrome *b* and DPNH are oxidized (II and III respectively).

In our laboratory an attempt has been made to ascertain whether the concentration of ~P acceptor is a rate controlling factor at each of the phosphorylation steps. Dr. Gladys Maley has found that ~P acceptors do not significantly enhance the rate of ascorbate or adrenaline oxidation in the system described previously (23). This could be interpreted to mean that the phosphorylation at site I is not tightly coupled with cytochrome oxidation. However, this system contains $10^{-4}$ M added cytochrome *c* — the agent that reacts with the substrate. It is possible that the oxidation of external cytochrome *c* is not as tightly coupled with phosphorylation as is the oxidation of the cytochrome *c* which is an integral part of the mitochondrial structure.

The combined regulatory effect of phosphorylation sites I and II is presumably observable in mitochondria oxidizing a substrate such as succinate, a process which proceeds with a P : O value of 2. With any given batch of mitochondria, ~P acceptor enhances oxidation of DPN-linked substrate more than of succinate. This indicates that phosphorylations at sites (I) and (II) are not as tightly coupled as is the DPNH phosphorylation (III).

Oxidation of pyridine nucleotide-linked substrates such as glutamate, β-hydroxybutyrate and citrate is strikingly dependent on ~P acceptor concentration (13, 28). In many experiments no oxygen consumption can be detected during the first 10 minutes of measurement in the absence of glucose and hexokinase. After the induction period a slow consumption is observed while the system with hexokinase and glucose consumes oxygen at a steady rate from the beginning.

As a consequence of the tight coupling of phosphorylation with oxidation of DPNH, the pyridine nucleotides



would be expected to be largely reduced in the resting state (29). This prediction was borne out by the experiments of Chance and Williams (18) who found that the reduced pyridine nucleotide became more oxidized upon addition of a phosphate acceptor such as ADP (18, 19). In some experiments done in collaboration with Drs. Chance and Williams in June of 1954, special precautions to obtain undamaged mitochondria resulted in preparations which, in the absence of acceptor, had virtually 100 % of their pyridine nucleotide in the reduced form.

Recently in our laboratory we have been studying phosphorylations associated with the mitochondrial oxidation of DPNH which is continually generated by ethanol and yeast alcohol dehydrogenase. This oxidation proceeds with P:O ratios of 2.5 to 2.7 indicating that externally reduced DPN may be as efficiently coupled with phosphorylation as that reduced within the mitochondria by D-β-hydroxybutyrate. Lehninger (30) has found that this system works well only in hypotonically treated mitochondria. In our laboratory such treatment has not been found necessary, possibly because we use sucrose to make up much of the tonicity in our reaction mixture. Oxygen consumption of mitochondria with the external hydrogen donor is considerably enhanced by $\sim$P acceptors but usually the effect is not as great as with β-hydroxybutyrate as the substrate.

A fourth phosphorylation site at the ' substrate level in the oxidation of α-ketoglutarate and triose-phosphate appears to be less tightly coupled than that at site (III) for in the absence of $\sim$P acceptor, α-ketoglutarate is usually oxidized at a more rapid rate than glutamate or other pyridine nucleotide linked substrates.

*Role of energy coupling in maintenance of reducing agents in cytoplasm and in reductive syntheses*

The cytoplasm of living cells contains an abundance of reducing agents such as ascorbic acid and glutathione and in some cases ergothioneine. These compounds are readily oxidized by molecular oxygen in the presence of a wide variety of tissue components (heme, the cytochrome system, catalase, phenol oxidases, and heavy metal salts). Despite this fact, the ascorbic acid and glutathione of tissues is preponderantly in the reduced form (31). It is very likely that they are maintained in that form because of the influence of the energy coupling in keeping the pyridine nucleotides reduced. The latter react with oxidized glutathione through specific reductases (32) to continually generate reduced glutathione and the latter reacts spontaneously and enzymatically to maintain protein-bound thiols and also ascorbic acid in their reduced forms.

The synthesis of fatty acids from carbohydrate must also depend upon the maintenance of hydrogen carriers in the reduced form (29). Both the reduction of β-keto acid to β-hydroxy (by DPNH, 33), and the reduction of the α-β unsaturated compound (by flavoprotein, 34), require two atoms of hydrogen. These are readily available when respiration is blocked by lack of $P_i$ or $\sim$P acceptor. It would appear that any decrease in the efficiency of oxidative phosphorylation would make less hydrogen available for such reductions. An exactly opposite deduction has been made by Hoch and Lipmann (35).

*Uncoupling of phosphorylation from oxidation*

In 1945 a consideration of the metabolic effects of 2,4-dinitrophenol (DNP), azide, gramicidin, and related agents led us to propose that « those agents which speed up metabolism and at the same time decrease the energy available for work or assimilation act by allowing oxidations to occur without phosphorylation or actually cause dephosphorylation of high energy phosphate » (36). Experimental evidence (12, 37, 38) is in agreement with this hypothesis although to this day the mechanism by which these agents work is not understood. DNP causes mitochondria to hydrolyze ATP (36, 38-40). Other treatments (heat, exposure to surface-active agents, silver ions, etc.) which result in disorganization of the mitochondrial structure also cause increased rates of ATP hydrolysis. Such effects can be clearly differentiated (41) from that of DNP which acts immediately, reversibly and which is active even with a solubilized preparation from mitochondria (40).

The most reasonable explanation of the action of DNP is that it causes the hydrolysis of an ' energy-rich ' bond in one of the intermediates of the oxidative phosphorylation machinery. This is represented in the following diagram modified from Slater (42) :

$$(1)\quad AH_2 + B + C \rightleftarrows A \sim C + BH_2$$

$$(2a)\quad A \sim C + P_i \rightleftarrows \begin{cases} A \sim P + C \text{ or} \\ C \sim P + A \end{cases}$$

$$(2b)\quad A \sim P \text{ (or } C \sim P) + ADP \rightleftarrows A \text{ (or } C) + ATP$$

$$(3)\quad A \sim C + H_2O \rightarrow A + C$$

$$(3a)\quad A \sim P \text{ (or } C \sim P) + H_2O \rightarrow A \text{ (or } C) + P_i$$

It is possible that $A \sim C$ reacts with ADP to form $A$ (or $C$) $\sim$ ADP. However, a reaction with $P_i$ seems more likely in view of the finding that in the presence of mitochondria, $P_i$ loses $^{18}O$ much more rapidly than $P_i$ is fixed (43). The reversibility of reaction *2a* could account for this exchange of $^{18}O$, and reversal of both *2a* and *2b* might account for the exchange of $^{32}P_i$ with ATP which occurs without net $P_i$ uptake (44). DNP might react with either of the above high-energy phosphate intermediates or with $A \sim C$ to form $A \sim$ DNP (or $C \sim$ DNP) a labile compound which might spontaneously hydrolyze. In the presence of a sufficiently high concentration of DNP, the concentration of $A \sim C$ would be decreased and hence less $^{18}O$ exchange (43, 44) would occur by reaction *2a*. Stimulation of ATP hydrolysis by DNP could be brought about by the reversal of reaction *2b* followed by the reaction of DNP with the $\sim$ intermediate thus formed. In mitochondria damaged by heat, surface agents, etc., reactions *3* and *3a* proceed rapidly.

Respiration of cells is therefore stimulated by DNP because the latter eliminates the regulatory influence of the limiting concentrations of $P_i$ and $\sim$P acceptor. Interference with the synthesis of $\sim$P is a sufficient explanation of the effect of DNP in depressing work-performance while stimulating respiration. In high concentrations, DNP depresses respiration, probably because it results in extensive hydrolysis of the nucleo-

tides and coenzymes which are a part of the respiratory machinery.

The similarity of the activated phenolic group of dinitrophenol and that of thyroxine has prompted comparisons of the effects of these two compounds on tissue metabolism (14, 45-50). It is now generally agreed that the deleterious and toxic effects of excessive amounts of thyroid hormone may result, at least in part, from its uncoupling activity (14, 47-51, 35). However, it has been our contention that the salutary effects of the thyroid hormone are also brought about by its uncoupling (51, 52). The fact that muscular work is performed with less net energy expenditure by the hypothyroid subject than by the normal person (53-55) indicates that even the minute amounts of thyroid hormone normally occurring in muscle affect the energy-coupling mechanism. The theory predicts that, at physiological concentrations, the thyroid hormone should uncouple only one of the phosphorylation sites in the electron transport sequence and that one should occur just prior to a rate-limiting step in the steady-state rate of oxidative phosphorylation. Martius (56) also favors a single site of action while Hoch and Lipmann (35) presented arguments in support of a general uncoupling activity. Dr. Gladys Maley and I (27) have studied the site of action of the hormone by examining the efficiency of individual phosphorylation steps. Liver mitochondria from rats which had been fed desiccated thyroid for 1 to 2 weeks oxidized glutamate, α-ketoglutarate, or D-β-hydroxybutyrate with P:O ratios of about 1 unit less than that obtained with mitochondria from normal rats. In these hyperthyroid mitochondria the phosphorylations occurring at sites I and IV were measured individually and found not to be significantly uncoupled. With ferricyanide as electron acceptor and β-hydroxybutyrate as substrate, phosphorylations II and III are measured. Mitochondria from hyperthyroid animals are only half as efficient as those from normal animals in coupling phosphorylation with reduction of ferricyanide (P:2 $Fe(CN)_6^{---}$ = 1.3 and 0.68 respectively). Attempts are being made to study these sites independently to define more clearly the action of the thyroid hormone. Certain other hormones might also function by disrupting an energy-coupling step (not necessarily involving phosphorylated compounds). For example, the adrenal cortical hormones seem to influence the interconversion of amino acids and proteins by accelerating reaction *b* :

$$\text{Amino acids} \underset{b}{\overset{a}{\rightleftarrows}} \text{Protein}$$

The parathyroid hormone might uncouple a reaction involved in phosphate resorption by the kidney. Martius (57) has studied the role of vitamin K in oxidative phosphorylation and the uncoupling effect of the anticoagulant dicumarol.

Cellular constituents other than vitamins and hormones influence the rate of oxygen consumption by mitochondria. Both the nuclei and microsome fraction of cells enhance respiration (58) presumably by increasing the rate of $P_i$ liberation from ATP (59). Microsomes contain a heat-stable, acetone-soluble substance which enhances respiration and uncouples phosphorylation by mitochondria (60). This has been identified (41) as a mixture of free fatty acids.

Because of the great variety of factors which might influence phosphorylation efficiency in the cell it would be of great importance to be able to measure P:O values in whole cells. Lynen and his students (61) have conducted such experiments with microorganisms but the difficulties are so formidable that no one has attempted similar studies with animal cells.

### *Influence of K and Mg on energy coupling*

The role of K in oxidative phosphorylation was reviewed four years ago (62). This ion has a profound effect on phosphate uptake by muscle mince (63) and liver mitochondria (41). With liver mitochondria the effect is more pronounced if incubation is prolonged (60, 62) or if agents harmful to the mitochondria are added. In table II (data from 41) are shown the results of experiments in which all-$Na^+$ or all-$K^+$ (60) media were compared. In the absence of ~P acceptors there is no requirement for added $K^+$. Stimulation of respiration by hexokinase, microsomes or surface active agents such as deoxycholic acid and fatty acids from microsomes makes apparent the requirement for added $K^+$. However when DNP is the agent which stimulates respiration, K seemed not to be necessary. Treatment with DNP, deoxycholic acid or fatty acids was found to deplete the mitochondria of their internally bound K (64).

These results may indicate that $K^+$ is not needed for reaction *1* (in the Slater scheme above) or for the interaction of DNP with the ~ intermediate that is formed. When respiration is stimulated by the surface active agents reactions *2a* and *3* may also be involved. Respiration and phosphate fixation in the presence of hexokinase requires reactions *1*, *2a* and *2b*. It therefore appears that $K^+$ is required for the reaction or group of reactions represented by *2a* or *2b*.

It has been known for some time that $Mg^{++}$ is essential for many oxidation systems and it is especially required to obtain maximum P/O values (12). Since $Mg^{++}$

TABLE II

*Effect of agents which stimulate mitochondrial respiration on the $K^+$ sensitivity of mitochondria*

| Agent added | $Qo_2$ (N) | | % stimulation by $K^+$ |
|---|---|---|---|
| | $Na^+$ system | $K^+$ system | |
| None | 74 | 72 | —3 |
| DNP, $3.3 \times 10^{-5}$ M | 250 | 248 | —1 |
| Deoxycholic acid, 0.1 mg. | 128 | 160 | 25 |
| Microsomes, 0.9 mg. N | 204 | 296 | 45 |
| Acetone extract of microsomes | 85 | 235 | 176 |
| Hexokinase | 157 | 243 | 55 |

The substrate was α-ketoglutarate and the $Qo_2$ (N) is recorded for the first 30 minutes of the experiments.

appears to be required for every transphosphorylation involving ATP and ADP (62) it probably participates in the reaction represented by *2b*. Added $Mg^{++}$ is necessary for maximum rates of succinate oxidation by mitochondria in the presence of hexokinase (fig. 4), it does not

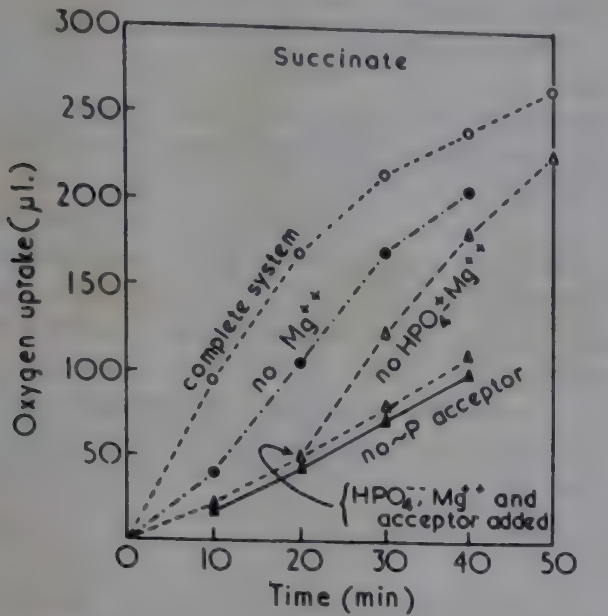



**Fig. 4.** — Effect of phosphate acceptor and $Mg^{++}$ on rate of succinate oxidation by rat liver mitochondria. Conditions as in figure 2 except as indicated. The complete system contained hexokinase and glucose as the acceptor.

influence the rate of respiration in the absence of ~P acceptor or in the absence of $P_i$. In all cases uptake of $P_i$ was dependent upon added $Mg^{++}$.

Thus a great variety of factors have been shown to influence rates of oxidation by mitochondria. It will certainly be necessary to know more about the mechanisms by which $P_i$ is fixed during electron transport for a better understanding of the regulatory processes. A very important aspect of this problem which has been neglected is the study of whole cells. Some of these, *e.g.*, spermatozoa, are particularly suited to problems of the kind discussed here (66, 67). Finally, it will be necessary to extend studies of the metabolism of whole organs under various conditions of work as has been done, for example, by Asmussen *et al.* (5, 6).

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## Diskussion zum Vortrag H. A. Lardy : « Energetic coupling and the regulation of metabolic rates »

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(*Eingegangen am* 17 *November* 1955)

Dr. Lardy hat uns soeben einen sehr schönen und umfassenden Ueberblick über die Rolle des anorganischen Phosphats und der Phosphatacceptoren bei der Regulation des Zellstoffwechsels gegeben. Ich möchte ihm, zugleich auch im Namen des Auditoriums, herzlich dafür danken.

Zur Steuerung von Atmung und Gärung bedient sich die lebende Zelle offensichtlich des einfachsten und zugleich wirkungsvollsten Mechanismus, den man sich dafür denken kann, indem sie das gleiche chemische System : (*a*) für die Ueberführung von Energie und (*b*) als Regulans einsetzt. Vergleichbar einem Akkumulator pendelt das ATP-System zwischen dem geladenen und entladenen Zustand hin und her, wobei normalerweise Atmung und Gärung, die das System unter Bildung von ATP aufladen, nur dann möglich sind, wenn anorganisches Phosphat und ADP, die Komponenten des entladenen Systems, verfügbar sind. Die Dissimilationsvorgänge kommen deshalb zum Stehen, sobald Orthophosphat oder ADP aufgezehrt sind.

Es ist darauf hinzuweisen, dass diese Koppelung zwischen Dissimilation und Phosphorylierung eine unmittelbare Konsequenz des in der Zelle realisierten Vorgangs der Energieüberführung ist. Wenn dort die Energie chemischen Reaktionsvermögens durch einen materiellen Träger transportiert werden kann, so ist das nur möglich, weil dieser Träger sowohl in der chemischen Reaktion des « Energie anliefernden Prozesses » wie auch in derjenigen des « Energie aufnehmenden Prozesses » stöchiometrisch teilnimmt. D.h., in den chemischen Gleichungen, durch welche beide Vorgänge beschrieben werden können, müssen ADP und Orthophosphat bzw. ATP als wesentliche Teilnehmer enthalten sein.

Unter diesem Gesichtspunkt kommt dem Studium der Wirkungsweise entkoppelnder Agentien allergrösste Bedeutung zu. Die Behauptung, dass die Kenntnis der chemischen Reaktion, durch welche Energie anliefernder Prozess und Phosphorylierung entkoppelt werden können, zum Verständnis des betreffenden Phosphorylierungsvorgangs verhilft, ist kaum übertrieben und kann überdies mit dem Hinweis auf die Untersuchungen von Warburg und Christian (1), in denen die entkoppelnde Wirkung des Arsenats bei der Gärung aufgeklärt wurde, belegt werden. Daher ist es verständlich, dass man sich im Anschluss an die Entdeckung der Wirkung des 2,4-Dinitrophenols auf die Atmungskettenphosphorylierung mit dem chemischen Mechanismus, der dieser Wirkung zugrundeliegt, eingehend beschäftigt hat. Ueber die dabei bisher erzielten Ergebnisse hat uns Dr. Lardy soeben berichtet.

In diesem Zusammenhang möchte ich Ihr Augenmerk auf eigene Experimente lenken, die unter Verwendung von intakten Hefezellen dem Problem der Wirkung von

Dinitrophenol gewidmet waren. Uns interessierte die Frage, ob der als « Entkoppelung » beobachtete Effekt des Dinitrophenols möglicherweise durch eine Aktivierung der Dephosphorylierung zustandekommt. In Untersuchungen an isolierten Mitochondrien ist ja gezeigt worden (2, 3, 4), dass die Geschwindigkeit der Spaltung von zugesetztem ATP durch Dinitrophenol eine wesentliche Steigerung erfährt. Wir benützten für unsere Experimente Hefezellen, die in glucosehaltiger Lösung atmeten und hinsichtlich des Phosphatkreislaufs einen stationären Zustand erreicht hatten, wo sich Phosphorylierung und Dephosphorylierung die Waage halten. Werden in diesem Zustand Atmung und Gärung mit 0.1 M Blausäure vergiftet, so kommt die Phosphorylierung zum Stehen. Die Dephosphorylierung läuft aber weiter und kann durch die Bestimmung des freigesetzten anorganischen Phosphats verfolgt werden.

In Versuchen an gärenden Hefezellen liess sich zeigen, dass diese Methode zuverlässige Werte liefert, denn es wurde dabei das erwartete P : $CO_2$ Verhältnis = 1 gefunden (5). Wir untersuchten daraufhin die Wirkung von Dinitrophenol auf die Dephosphorylierung (6). In einer Reihe von Experimenten, aus welcher ein Beispiel in Abbildung 1 wiedergegeben ist, ergab sich, dass die

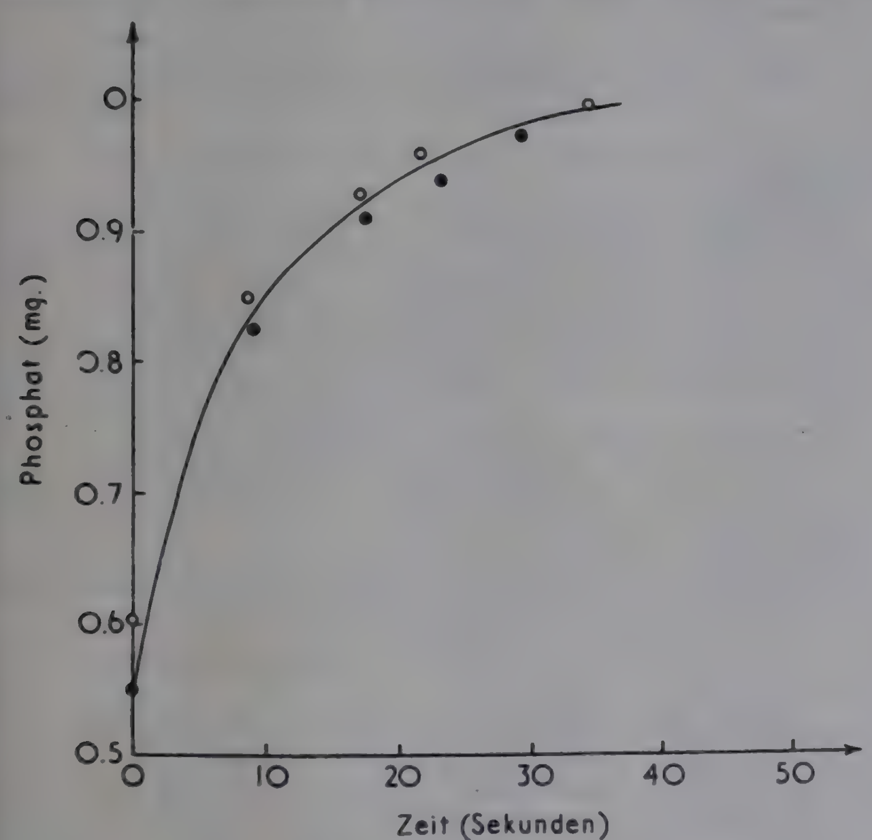


ABB. 1. — Dephosphorylierung mit und ohne 2,4-Dinitrophenol (6). 2 % Hefesuspension mit 0.4 % Glucose versetzt und 15 Minuten lang mit $O_2$ durchströmt. Zur Zeit 0 je 50 ml. mit 6 ml. M HCN + 6 ml. 2 mM Dinitrophenol (●) bzw. + 6 ml. $H_2O$ (○) versetzt. Die angegebenen Phosphatwerte beziehen sich auf. 1 g. Hefe feucht.

Kinetik der Bildung von Orthophosphat durch Dinitrophenol nicht verändert wird, die entkoppelnde Wirkung der Nitroverbindung also sicher nicht durch eine Aktivierung der Dephosphorylierung zustandekommen kann.

Diese « Blausäure-Methode » haben wir weiterhin zur Bestimmung der P : O Quotienten in lebenden Hefezellen benutzt. Da ja im stationären Zustand Phosphorylierung und Dephosphorylierung einander gleich sind, gibt die nach Zugabe von Blausäure messbare Dephosphorylierung Auskunft über die Phosphorylierungsgeschwindigkeit. Ueber Versuche an in Glucoselösung atmenden Hefezellen habe ich zusammen mit Königsberger (6) vor vier Jahren berichtet. Als P : O Quotient wurde ein Wert von etwa 1 gefunden. Neuerdings untersuchte Netter (7) in meinem Laboratorium auch den Wirkungsgrad der endogenen Atmung von Hefezellen. Dieser Atmungsprozess, bei welchem Reservestoffe der Hefe einer langsamen Verbrennung anheimfallen, läuft in Gegenwart eines wesentlich höheren Phosphatniveaus innerhalb der Zelle ab. Wie Tabelle I zeigt, in der die

TABELLE I

*P : O Quotient der endogenen Atmung von Hefezellen* (7)

| Dephosphorylierungs-geschwindigkeit (µ-Mole $PO_4^{3-}$/30 Sek./200 mg. trocken Hefe) | Atmung (µ-Atome O/30 Sek./200 mg. trocken Hefe) | P : O Quotient |
|---|---|---|
| 0.75 | 0.345 | 2.12 |
| 0.57 | 0.300 | 1.90 |
| 0.474 | 0.225 | 2.11 |
| 0.647 | 0.357 | 1.81 |
| 0.73 | 0.302 | 2.42 |
| 0.615 | 0.352 | 1.75 |
| 0.592 | 0.273 | 2.17 |
| 0.698 | 0.347 | 2.01 |
| 0.710 | 0.309 | 2.30 |
| 0.525 | 0.291 | 1.80 |
| 0.905 | 0.288 | 3.14 |
| 0.588 | 0.324 | 1.81 |
| 0.614 | 0.271 | 2.27 |
| 0.525 | 0.261 | 2.01 |
| 0.58 | 0.250 | 2.32 |

Zu den Versuchen kam 1 Stunde lang verarmte Bäckerhefe (Hefefabrik Oberkotzau) in Anwendung, die in 0.02 M Citratpuffer, pH 5.4, unter Luft geschüttelt wurde. Hefetrockensubstanz : 14-16 mg./ml. Suspension.

$O_2$-Verbrauch : manometrisch in der Warburg-Apparatur ermittelt.

Dephosphorylierungsgeschwindigkeit : Messung vergleiche (6). Die Hemmung der Phosphorylierung wurde durch Zugabe von 1/10 Vol. M HCN-Lösung erreicht.

Temperatur : 15° C.

Ergebnisse der neuen Versuche aufgeführt sind, liegt der P : O Quotient der endogenen Atmung durchschnittlich bei 2.1, in einigen Fällen sogar in der Nähe von 3, dem, den Versuchen an tierischen Mitochondrien zufolge, theoretischen Maximalwert. Offensichtlich hat also auch der Einzeller die Möglichkeit, den Wirkungsgrad der Atmung zu variieren. Für den Fall, dass nur wenig Brennmaterial zur Verfügung steht (endogene Atmung), muss die Zelle bestrebt sein, die bei der Oxydation entbundene chemische Energie möglichst weitgehend in verwertbare Phosphatenergie einzufangen, während bei reichlichem Angebot an Nahrungsstoffen (Atmung mit zugesetzter Glucose) auch schon ein geringerer P : O Quotient den Energiebedarf der Zelle decken kann. Zweifellos ist die Erniedrigung des P : O Quotienten in Gegenwart von Zucker biologisch sinnvoll. Denn es ist zu bedenken, dass die Zellen ja unter diesen Bedingun-

gen wachsen und sich vermehren können und deshalb ausser Energie auch Baumaterial benötigen. Je geringer aber der P : O Quotient ist, umso grösser muss die Atmung der Zellen sein, damit der Bedarf an Phosphatenergie gedeckt wird. Umso höher wird aber damit die stationäre Konzentration an den Zwischenprodukten der Atmung, wie Acetyl-CoA, Oxalacetat oder α-Ketoglutarat, deren sich der Organismus beim Aufbau von neuem Zellmaterial bedienen kann.

Zuletzt noch einige Bemerkungen zum Pasteur-Effekt, jenem biologisch überaus zweckmässigen Phänomen der Gärungshemmung durch die Atmung. Der Pasteur-Effekt hat zur Folge, dass die lebende Zelle unter aeroben Bedingungen, wo ihr bei der vollständigen Oxydation der organischen Brennstoffe die Entbindung der gesamten potentiellen chemischen Energie möglich ist, weniger Material umsetzt als bei Ausschluss von Sauerstoff, wo auf dem Wege der Gärung nur ein meist kleiner Teil dieser Energie freigesetzt werden kann.

Im Jahr 1941 berichtete ich über Experimente an Hefezellen, wonach die Ursache des Pasteur-Effektes im Phosphatkreislauf zu suchen ist (8). Unter Sauerstoff kommt es zur Konkurrenz von Atmung und Gärung um Orthophosphat und ADP, die unter anaeroben Bedingungen ausschliesslich dem Gärungsvorgang zur Verfügung stehen. Nimmt man an, dass zwischen aerober und anaerober Dephosphorylierung praktisch kein Unterschied besteht — was von vornherein sehr wahrscheinlich war und sich in einer späteren Arbeit auch experimentell beweisen liess (6) — so ist die vom Dazwischentreten des Sauerstoffs ausgelöste Gärungshemmung sofort verständlich. Jetzt müssen sich Atmung und Gärung in die durch die Geschwindigkeit der Dephosphorylierung begrenzte Phosphorylierung teilen, so wie das in Abbildung 2, links schematisch wiedergegeben ist. Rechts ist der Phosphatkreislauf in der anaeroben Zelle dargestellt.

Der eigentliche Anlass zur Hemmung der Gärung durch die Atmung ist gemäss dieser Vorstellung eine Erniedrigung der in der Zelle frei vorliegenden Orthophosphat und ADP, der Angriffspunkt in der Fermentkette der Gärung demnach : die Dehydrierung des Triosephosphats

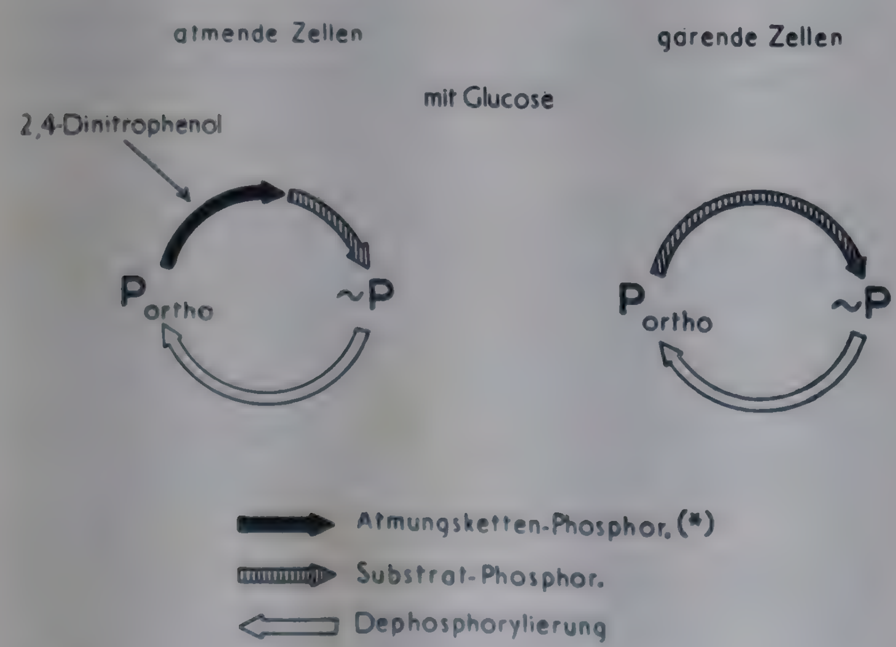


ABB. 2. — Phosphatkreislauf in Hefezellen.

(*) Zur Unterscheidung von Atmungsketten- und Substrat-Phosphorylierung (vgl. 6 und 9).

und die Bildung von 3-Phosphoglycerat aus der Negelein-Säure (Glycerinsäure-1,3-diphosphat).

Diese Vorstellung, zu welcher unabhängig von uns auch Johnson (10) gekommen war, steht in Uebereinstimmung mit einer Reihe experimenteller Befunde. So hatten wir bei Versuchen mit Hefe gefunden, dass aerob weniger anorganisches Phosphat vorliegt als anaerob (8, 6). Ausserdem enthalten die Zellen, nach neuen Experimenten von Holzer (11), unter Sauerstoff mehr ATP und weniger ADP. Holzer (12) fand in aerober Hefe auch mehr Hexosediphosphat und Triosephosphat, wobei noch besonders zu erwähnen ist, dass Fructosediphosphat mit den Triosephosphaten im thermodynamischen Gleichgewicht steht, so dass eine Theorie, die den Pasteur-Effekt über eine Hemmung der Aldolase erklären will (13), nicht zutreffen kann. Für die Mitwirkung des Phosphatkreislaufs spricht aber vor allem auch die Beobachtung, dass der Pasteur-Effekt durch Dinitrophenol und andere Entkoppelungsgifte aufgehoben wird (6, 14-17). Wenn in Gegenwart dieser Gifte beim Wasserstoff-bzw. Elektronentransport über die Atmungskette keine Phosphorylierung erfolgt, dann kann der Theorie zufolge die Atmung auch keine Wirkung mehr auf die Gärung haben. Im Phosphatkreislauf unterscheiden sich solche Zellen nicht mehr von anaeroben Zellen : Die Atmungskettenphosphorylierung verschwindet, so dass dann auch in Gegenwart von $O_2$ die Dephosphorylierung praktisch vollständig (*) dem Ausgleich der Phosphorylierung bei der Gärung dienen kann (vgl. Abbildung 2).

Wenn diese Theorie somit den wesentlich reduzierten Abbau des Zuckers unter Sauerstoff zwanglos erklären kann, so darf nicht übersehen werden, dass bei der Deutung der verringerten Resorption des Zuckers unter aeroben Bedingungen Schwierigkeiten auftauchen. Bestimmt man den Glucoseschwund im umgebenden Medium, so findet man, dass atmende Hefezellen nur etwa halb so schnell Zucker verbrauchen als gärende. Für diese Tatsache, die seit langem bekannt ist (18), bringt Abbildung 3 einen Beleg.

Wir müssen deshalb annehmen, dass die von Hexokinase katalysierte Phosphatübertragung :

$$\text{ATP} + \text{Glucose} \rightleftarrows \text{ADP} + \text{Glucose-phosphat},$$

mit welcher der Umsatz des Zuckers in der Hefezelle eingeleitet wird, anaerob beinahe doppelt so schnell erfolgt wie aerob. Im Hinblick auf die durch den Atmungsprozess ermöglichte zusätzliche Bildung von ATP würde man das Umgekehrte erwarten und tatsächlich findet sich in Hefezellen auch aerob mehr ATP als anaerob (11).

Zur Erklärung dieses Befundes könnte man annehmen, dass die Hexokinase durch Sauerstoff direkt gehemmt wird. Doch hat man beim Studium des isolierten Enzyms bisher nichts Derartiges beobachtet und ausserdem liesse sich dann die Wirkung von Dinitrophenol nicht erklären, in dessen Gegenwart der aerobe Zuckerverbrauch auf den anaeroben Wert ansteigt (14, 15, 19).

Eine andere Erklärung wäre, dass die Hexokinase durch Hexosephosphate gehemmt wird, die sich in der Zelle anhäufen, weil der weitere Umsatz über die Stufe

(*) Im Citronensäure-Cyklus kommt es ja dann nur noch bei der Spaltung von Succinyl-CoA zur Bildung von ATP (29, 30).

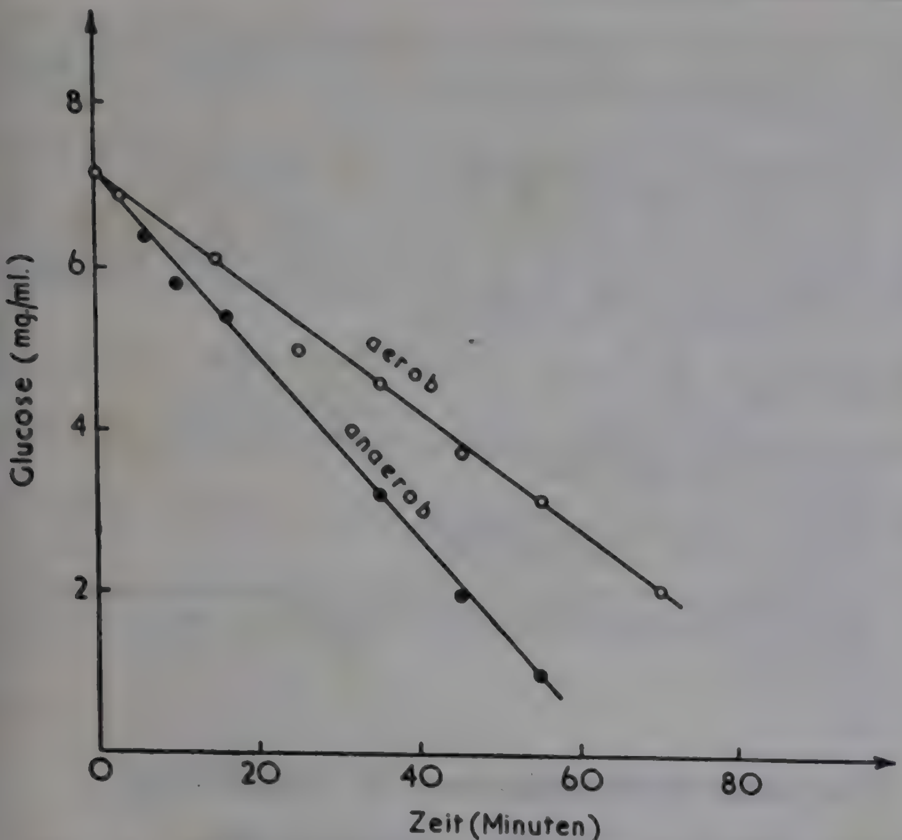


ABB. 3. — Zuckerverbrauch aerob und anaerob (19). 2 g. Bäckerhefe (580 mg. trocken) in 50 ml. 0.02 M Citratpuffer, pH 5.4, der 0.72 g. Glucose/100 ml. enthält. Aerob (○) : unter $O_2$; anaerob (●) : Atmung durch Zugabe von 2 mM HCN gehemmt. Temperatur : 25° C. Glucosebestimmung nach Hagedorn-Jensen, modifiziert von Garbade (31).

des Triosephosphats, wie wir vorhin erfahren haben, teilweise blockiert ist (6). Beim Studium der Hexokinase aus tierischen Geweben beobachteten Weil-Malherbe und Bone (20), sowie Crane und Sols (21) tatsächlich eine starke Hemmung durch Glucose-6-phosphat, so dass also im Tierkörper eine solche Deutung zutreffen könnte. Die Hexokinase der Hefe wird von Glucose-phosphat jedoch nicht beeinflusst.

Wir zogen deshalb eine dritte Erklärung in Betracht (6), der die Beobachtung zugrundeliegt, dass Atmungs- und Gärungsenzyme auf verschiedene Bereiche der Zelle verteilt sind. Bei der Fraktionierung von Homogenaten tierischer Organe ergab sich, dass die Enzyme der Glykolyse im Cytoplasma, die Enzyme der Atmungskette und des Citronensäure-Cyklus hingegen in den Mitochondrien enthalten sind (22). Auch in Hefezellen scheinen Gärungs- und Atmungsenzyme auf verschiedene Bereiche der Zelle verteilt zu sein (23). Das hat zur Folge, dass die Bildung des ATP bei der Gärung im Cytoplasma, bei der Atmung hingegen fast ausschliesslich in den Mitochondrien erfolgt. Ausserdem weiss man heute, dass Mitochondrien eine Membran besitzen und auf Kosten der Atmung Ionen im Innern zu speichern vermögen (24-27). Man kann sich deshalb vorstellen, dass atmende Mitochondrien über die Atmungskettenphosphorylierung ATP konzentrieren können. Dann würden mit dem Einsetzen der Atmung die Adeninnukleotide aus dem Cytoplasma an die Mitochondrien wandern und folglich die im Cytoplasma lokalisierte Hexokinase an ATP verarmen.

Mit dieser Vorstellung liesse sich erklären, warum Hefezellen unter Sauerstoff weniger Glucose aufnehmen als unter Stickstoff, und warum Dinitrophenol den Glucoseverbrauch auf den anaeroben Wert erhöht. Dass in atmenden Hefezellen eine Stoffwanderung an die strukturierten Elemente der Zelle erfolgt, geht aus einer Untersuchung hervor, die Holzer (28) vor fünf Jahren in meinem Laboratorium ausführte. Er plasmolysierte atmende, gärende oder « verarmte » Hefezellen durch Einfrieren in flüssiger Luft und trennte das aufgetaute Material anschliessend durch Zentrifugieren in eine, die Bestandteile des Zellsafts enthaltende « Lösung » und einen, aus den strukturierten Elementen der Zelle bestehenden « Bodensatz ». Er fand, dass bei kräftig atmenden Hefezellen ein wesentlich grösserer Prozentsatz des insgesamt vorhandenen Orthophosphats im « Bodensatz » lokalisiert war als bei gärenden oder gar bei den kaum noch einen Stoffwechsel aufweisenden « verarmten » Zellen. In diesem Ergebnis können wir den Beweis für die Existenz eines « Strukturphosphats » sehen, das wir seinerzeit als eine überauss labile, sowohl durch Erhitzen als auch durch Behandlung mit Trichloressigsäure momentan spaltbare Eiweiss-Phosphat-Verbindung ansahen. Heute möchte ich annehmen, dass dieses « Strukturphosphat » Orthophosphat war, das in Mitochondrien kumuliert vorlag. Diese Annahme findet eine starke Stütze in Versuchen, über welche Bartley und Davies (26) im vergangenen Jahr berichteten und in denen gezeigt wurde, dass Orthophosphat von isolierten Mitochondrien aus Schafsnieren beim Atmungsprozess gespeichert werden kann.

Die Verteilung von ATP auf « Lösung » und « Bodensatz » wurde von uns leider nicht bestimmt, so dass die oben vorgetragene Hypothese vorerst noch nicht experimentell belegt werden kann. Da aber Bartley und Davies (26) mit Hilfe der Isotopentechnik nachweisen konnten, dass das in den Mitochondrien gespeicherte Orthophosphat aus dem ATP des Mediums stammt, darf man wohl annehmen, dass auch ATP an den Strukturelementen der Zelle während der Atmung kumuliert wird. Aus all dem ergibt sich, dass man bei der Betrachtung intrazellulärer biochemischer Reaktionen der Topographie der Zelle Beachtung schenken muss. Die Beschäftigung mit den damit zusammenhängenden Fragen wird uns wahrscheinlich das Verständnis des Pasteur-Effekts in allen Einzelheiten vermitteln können.

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# The relationship of dinitrophenol activated adenosine triphosphatase to uncoupling mechanisms and possible phosphorylation of electron transfer catalysts (*)

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(*Received* 19 *September* 1955)

### *ATPase activation and uncoupling by DNP*

Like Dr. Lardy and others, we have for some years considered an intimate relationship between these two effects as being probable. However, Harman (1) and others have considered ATPase activation to be almost entirely a matter of structural alterations in mitochondria although there is little to suggest that uncoupling is a structural effect.

In studies on ATPase activation by various uncoupling agents we ourselves have made observations which might be taken as indicating little relationship between uncoupling and ATPase activation. However, at present we believe the data can be explained best on the basis that dinitrophenol (DNP) activated ATPase represents reversal of part of the reaction sequence involved in ATP formation by oxidative phosphorylation, with the site of action of DNP being identical with that for uncoupling. Some of these experiments will be discussed briefly.

First, the minimal concentration of DNP for complete uncoupling does not give complete activation of ATPase. The ATPase activity may be doubled by a five fold increase in DNP concentration over that which gives essentially 100 % uncoupling. This might be interpreted as action at two different sites. However, we must remember that there is no *a priori* reason why the concentrations producing the maximal effects should be the same. Within limits the rate of splitting of the intermediate ($Y \sim PO_4$) should depend on the concentration of DNP. As soon as this rate exceeds the rate of formation uncoupling should be 100 %, but if the reactions between $Y \sim PO_4$ and ATP are readily reversible, the splitting of ATP would continue to increase with the concentration of DNP.

A second observation is that other uncoupling agents give less activation of ATPase than DNP, sometimes much less. All uncoupling agents probably do not act by the same mechanism, but it is puzzling that one like gramicidin gives much less ATPase than DNP, for it closely resembles DNP in its great potency and selectivity as an uncoupler. This could be explained by two sites of action for DNP (uncoupling and ATPase) and only one for gramicidin (uncoupling). However, again there is no *a priori* reason why the rate of splitting of $Y \sim PO_4$ should be the same with each substance if DNP and gramicidin act by the same mechanism (figure 1).

In addition, low concentrations of gramicidin activate more ATPase than higher concentrations. This suggests that gramicidin inhibits one of the reactions in the

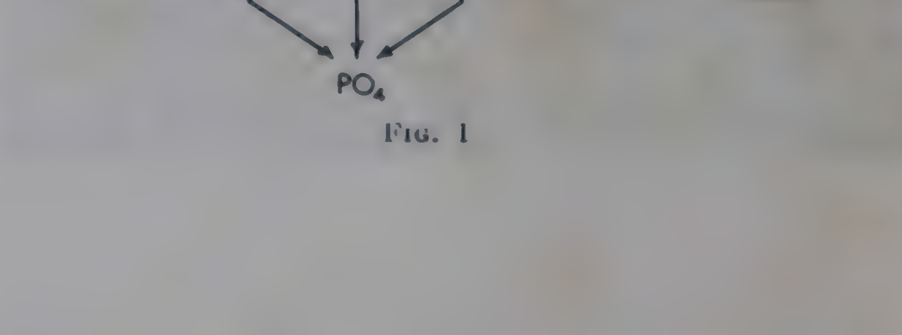


FIG. 1

(*) Aided by a grant from the United States Public Health Service.

sequence responsible for ATPase. This interpretation is greatly strengthened by the finding that the same concentrations of gramicidin also inhibit DNP activated ATPase (table I), but not DNP uncoupling. The inhibition by gramicidin is inhibition after activation, not inhibition of the activation process. Azide and p-chlormercuribenzoate behave qualitatively like gramicidin, activating some ATPase, but inhibiting DNP

TABLE I

*Dephosphorylation of ATP by mitochondria*

| Expt. nr. | Uncoupling agents | µ-moles phosphate split from ATP |
|---|---|---|
| 23*a* | None | 0.37 |
| | $4 \times 10^{-4}$ M DNP | 2.18 |
| | $2 \times 10^{-5}$ M *p*-CMB (*) | 0.68 |
| | $4 \times 10^{-4}$ M *p*-CMB (*) | 0.26 |
| | $4 \times 10^{-4}$ M DNP + $2 \times 10^{-4}$ M *p*-CMB (*) | 0.31 |
| 23*b* | Gramicidin (3 µg./ml.) | 1.10 |
| | Gramicidin (65 µg./ml.) | 0.52 |
| | $4 \times 10^{-4}$ M DNP + gramicidin (3 µg./ml.) | 2.39 |
| | $4 \times 10^{-4}$ M DNP + gramicidin (65 µg./ml.) | 0.79 |

(*) *p*-CMB = *para*chloromercuribenzoate.

activated ATPase. These observations are explainable in the hypothesis that gramicidin and certain other uncoupling agents inhibit transphosphorylation from ATP to Y, a process which must occur before ~P is split off (reaction *4*, figure 1). Since 85 % inhibition of the ATPase does not alter uncoupling by DNP, uncoupling is probably not secondary to ATPase activation.

### *ATPase activation by structural changes*

Many workers have observed increased ATPase after structural alterations in mitochondria. Undoubtedly the penetration of ATP to the site of splitting is one factor involved. For example, hypertonic sucrose causes a small increase in ATPase itself, but greatly reduces the activation by DNP. The changes under hypertonic conditions probably decrease permeability to ATP or interfere with transfer of phosphate to the site of DNP action.

Swelling and disintegration, regardless of how produced, increase ATPase activity. The maximum that we have observed is of the same order of magnitude as that with DNP. This is seen only within certain concentration ranges of surface active agents, as higher concentrations or prolonged physical treatment cause inhibition or inactivation. Simultaneously with the progressive activation of ATPase by structural alteration there is a decrease in the activation by DNP, so that the total ATPase often remains about the same. This may be coincidental, but at least equally probable is a relationship between the DNP activated ATPase and that appearing on structural disruption. However, this does not necessarily mean that the action of DNP is a structural one. In both cases the same enzyme system may be involved. Structural disruption may lead to alteration of certain groups which are protected in the intact system, so that the system now reacts with water, splitting ATP. The action of DNP in producing (directly or indirectly) a net reaction with water is characteristic of the native system, and it should not be surprising if DNP has no effect once the system is altered and already reacting with water at a maximal rate. DNP would act only on that portion of the system remaining in the native state.

Harman and coworkers (1) feel there is good correlation between structural changes and ATPase, even with DNP, but others have reached different conclusions (2). Price and Davies (3) have observed that low concentrations of DNP cause little or no swelling of mitochondria (confirmed in our laboratory), yet these concentrations are those which greatly activate ATPase. Furthermore DNP does not release acid phosphatase, which is released by physical disruption. Finally, reversibility of the action of DNP (4) suggests that there is no drastic structural change. Admittedly, minor changes could be reversible. Further experiments with the 'soluble' DNP activated ATPase (Lardy and Wellman, 5) should tell us whether the action of DNP involves structure.

### *Electron transfer prosthetic groups as possible phosphorylated intermediates*

Phosphorylated forms of electron transfer catalysts as intermediates in aerobic phosphorylation have represented an attractive hypothesis for some years. If they are involved and if DNP activated ATPase bears the postulated relationship to phosphorylation intermediates, the state of the electron carriers (oxidized or reduced) should determine the amount of ATPase seen with DNP. This is illustrated in figure 2, using the generalized formulation with A, B and C. A is active in electron transfer. If A ~ $PO_4$ were the intermediate the reverse reaction with ATP would require A. The amount of DNP activated ATPase should be small if A is reduced to $AH_2$ (substrate + anaerobic conditions). If something besides the electron transfer agent itself is the phosphorylated intermediate (for example C ~ P), reduction of A to $AH_2$ would not change DNP activated ATPase.

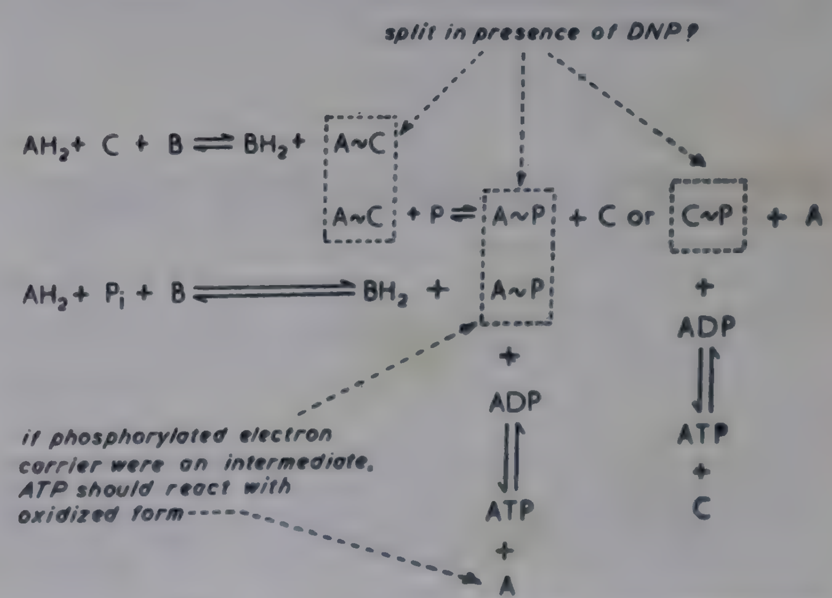


FIG. 2. — Possible relationship of DNP-ATP ase to the oxidation-reduction state of electron carriers.

Some years ago we determined the ATPase both anaerobically and aerobically. We were disappointed when the ratio between the two always came out to be about 0.6-0.7. Recently we have repeated these experiments with improved technique. The ratio still averages around 0.65, with a range 0.53-0.95. Although DNP activated ATPase was not reduced to low levels anaerobically, as we thought it might be, it was always lower than aerobically. One possible interpretation is that only one out of three sites involves a phosphorylated form of the electron transfer catalyst directly, and therefore only one is subject to modification by reduction.

Unfortunately a number of differences may occur under anaerobic conditions and we cannot be sure that even one electron transfer catalyst is a phosphorylated intermediate. Even though both aerobic and anaerobic experiments contained DNP in high concentration and we have added both $K^+$ and $Mg^{++}$ in most of the experiments, there might be differences in internal $K^+$ and $Mg^{++}$ concentrations.

### *Coupling may determine what is seen with inhibitors*

Finally, it is interesting to note how the closely coupled nature of the phosphorylation probably determines what will be observed when certain inhibitors are studied. In brain cortex slices with pyruvate as substrate narcotics in anesthetic concentrations produce only small inhibitions of respiration, but in electrically stimulated slices with doubled oxygen uptake the same concentrations produce much greater inhibition (6). Since stimulation by the uncoupling agent DNP produces the same picture as electrical stimulation it appears likely that in the unstimulated slice the rate limiting factor is the low level of ~P acceptors. This picture does not hold for succinate oxidation, so the step where the rate is limited by ~P acceptors is probably at the DPN level. Dr. Lardy has suggested that this is the most tightly coupled step.

A 50 % reduction of active enzyme (inhibition) in the narcotic sensitive region (figure 3) might have virtually no effect on oxygen uptake if the rate determining factor is ~P acceptor concentration at the DPN level. However, once this phosphate acceptor limitation is removed (DNP, or ATP utilization due to electrical stimulation) the point inhibited by narcotics may become the rate limiting step so the full inhibitory action of narcotics will be manifest.

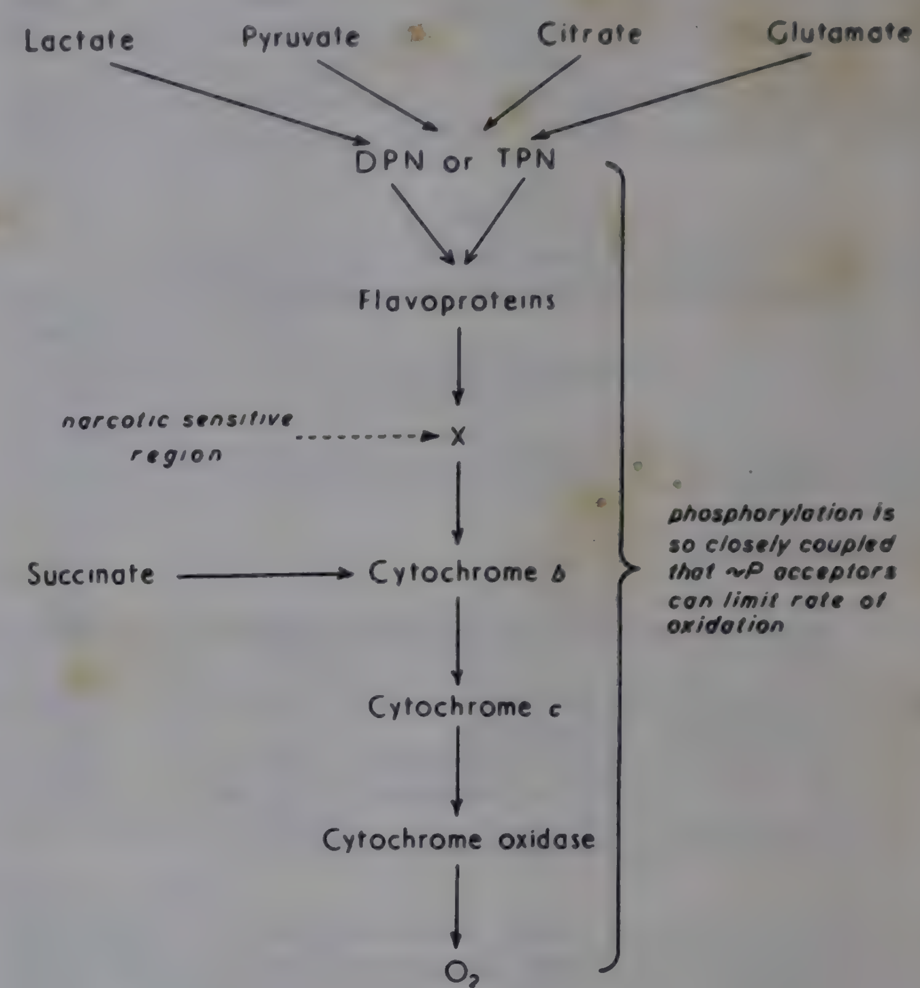


FIG. 3.


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## On possible mechanisms for the control of electron transport in the respiratory chain (*)


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(*Received* 20 *October* 1955)

By means of simultaneous measurements of increases in respiratory rates and of the oxidation-reduction levels of the electron transport component of phosphorylating liver mitochondria upon the addition of phosphate acceptor ADP (*), it has been possible to localize at least three components of the respiratory chain that are involved in oxidative phosphorylation : cytochromes *c* and *b* and DPNH (1-4). This paper reports studies with other reagents for affecting the oxidation-reduction levels and respiratory rates, calcium, magnesium, DNP,

(*) This research was supported in part by grants from the National Science Foundation and from the Office of Naval Research.

DBP (*), and dicoumarol, etc. On the basis of these and related experiments it has been possible to show that reagents causing loss of respiratory control appear to act at the same site, namely at DPNH, and, in their effect upon phosphorylation efficiency, these reagents act at three or more sites as does ADP. It is further possible to explain the relationship between phosphorylation efficiency and respiratory control.

*Methods and preparations.* — The spectroscopic (5) and polarographic (6) methods used in these studies have been described elsewhere. It may be sufficient to state here that the spectrophotometric method is especially designed to be insensitive to the optical artifacts that occur with mitochondria. The polarographic method can be used at the same time as the spectroscopic one and, in addition, responds rapidly enough to measure accurately the oxygen utilization caused by the addition of a given amount of ADP. The mitochondria are prepared by the method of Schneider (7) as modified by Lardy and Wellman (8) and show P : O values of about 3 for glutamate and β-hydroxybutyrate (6) and their respiratory control (ratio of the rates with and without ADP) may exceed 10 fold.

*Effects of ADP.* — In addition to the marked stimulation of respiration obtainable with these preparations upon the addition of ADP to mitochondria supplied with substrate, phosphate, magnesium. etc., there are distinctive changes in the oxidation-reduction levels of the cytochrome, flavo-protein, and pyridine nucleotide components of the respiratory chain. Cytochromes *b* and *c*, flavo-protein and DPNH become more oxidized as the respiration is increased by ADP addition (state 4 to 3 transition) (4) and become more reduced when the added ADP is exhausted (state 3 to 4 transition) (4). Thus in the state 4 to 3 transition one would expect the α-bands of cytochromes *c* and *b* at 550 and 565 mμ to be diminished, as is indicated by the spectroscopic record of figure 1 (upper trace). Note that decreases of optical density are plotted upwards in this graph.

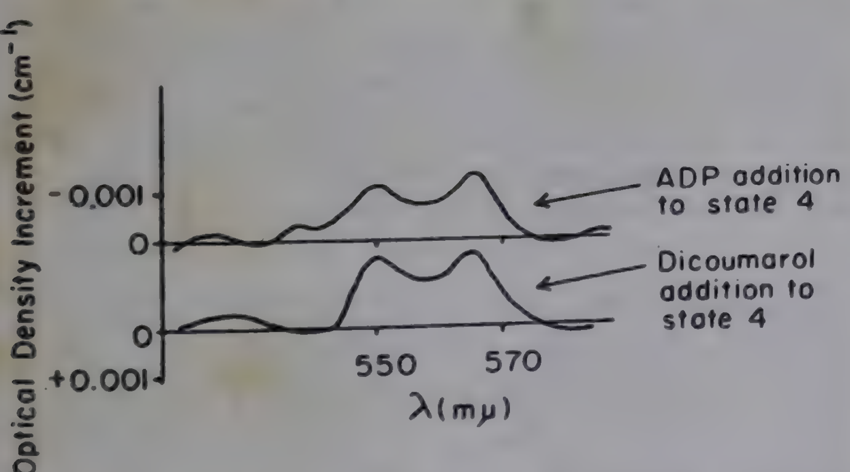


FIG. 1. — A comparison of the effects of ADP and dicoumarol upon the steady-state oxidation-reduction levels of cytochromes *b* and *c* (564 and 550 mμ respectively) in rat liver mitochondria. The base line of the two traces represents the mitochondrial suspensions with β-hydroxybutyrate as substrate, the curves represent the changes of optical density : a decrease being registered as upward deflection. Isotonic medium, 400 μM ADP, 14 μM dicoumarol (Expt. 468 *d*-6).

*Effect of uncoupling agents.* — If, instead of ADP an uncoupling agent such as dicoumarol is added, the same two peaks due to cytochrome *b* and *c* are recorded. Thus, as far as cytochromes *b* and *c* are concerned, the effects of ADP and dicoumarol upon the oxidative phosphorylation system are identical. And spectroscopic recordings at other wavelengths using DNP and DBP (*), show that DPNH and flavo-protein are also affected similarly. Thus these agents affect all sites of phosphorylation as does ADP. In the case of TIT and thyroxine, the need for a prolonged incubation period (9) leads to technical difficulties, but it is clear that both reagents affect the respiratory chain up to, and including, cytochrome *b*. Thus these data to not support the idea that these uncoupling agents, at these concentrations ($2 \times 10^{-5}$ M TIT), show a specificity for uncoupling phosphorylation at a particular member of the respiratory chain (10, 11); apparently they interact with at least two of the phosphorylation sites that ADP reacts with.

A possible mechanism for the action of such uncoupling agents at a site of phosphorylation, for example, cytochrome *c* would follow closely the reaction mechanism already proposed (4) : the oxidation-reduction reaction leads to the conservation of a portion of the free energy changes in a hypothetical compound $c'' \sim \mathrm{I}$. On the basis of data on the kinetics of the reaction of ADP and uncoupling agents with the respiratory chain, we find it necessary to postulate a rate-limiting reaction of $c'' \sim \mathrm{I}$ with an intermediate X

$$c'' \sim \mathrm{I} + \mathrm{X} \leftrightarrows \mathrm{X} \sim \mathrm{I} + c'' \qquad (1)$$

Our experiments data are consistent with the hypothesis that the compound $\mathrm{X} \sim \mathrm{I}$ can react with either ADP + $P_i$ or the uncoupling agents :

$$\mathrm{X} \sim \mathrm{I} + \mathrm{ADP} + \mathrm{P_i} \leftrightarrows \mathrm{X} + \mathrm{I} + \mathrm{ATP} \qquad (2)$$

$$\mathrm{X} \sim \mathrm{I} + \mathrm{DNP} \leftrightarrows \mathrm{X} + \mathrm{I\text{-}DNP} \qquad (3)$$

In the latter case the uncoupling agent can combine with the substance I so that none is available for the formation of ∼I compounds. Reactions similar to equations (*1*), (*2*) and (*3*) occur at the two others sites, DPNH and cytochrome *b*. Alternative mechanisms for equation (*3*) involve the hydrolysis of the $\mathrm{X} \sim \mathrm{I}$ compound.

*The effect of calcium.* — While the uncoupling effects of calcium are well-known, our data show early phases of the calcium effect that are rather different from its later effects, as illustrated by figure 2. In this record, one trace records oxygen utilization as an upward deflection (platinum microelectrode) and the other trace records optical density decreases at 340 mμ (with respect to 374 mμ) as an upward deflection which corresponds to an oxidation of DPNH. Addition of 135 μM ADP causes the rapid utilization of 20 μM oxygen and a simultaneous cycle of oxidation and reduction of DPNH. Addition of 385 μM calcium also causes rapid respiration

(*) The following abbreviations are used : ADP = adenosine diphosphate ; DNP = dinitrophenol ; DBP = dibromophenol ; TIT = triiodothyronine ; DPNH = dihydrodiphosphopyridine nucléotide. In the figures, M means molar (moles/liter).

(*) Experiments carried out in collaboration with Dr. G. R. Williams and with Dr. H. A. Lardy.

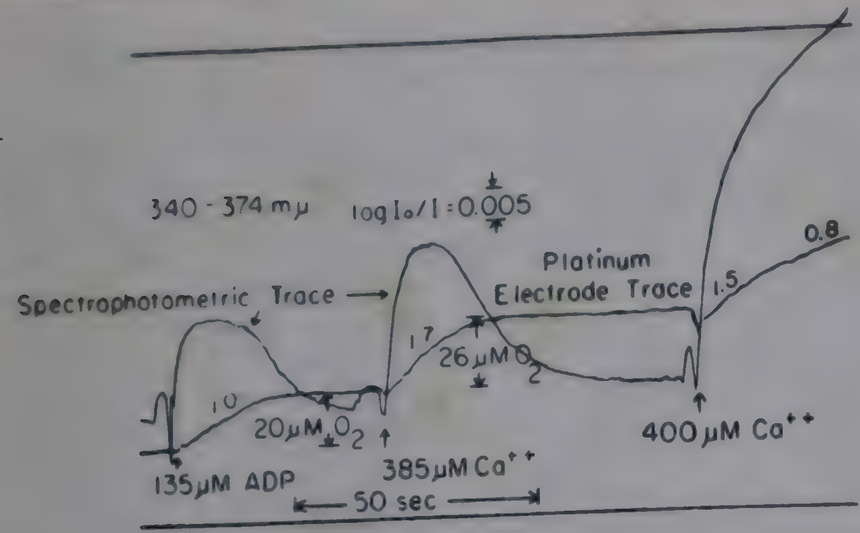


FIG. 2. — Simultaneous respiration and spectrophotometric recordings of the effects of ADP and calcium. Oxygen utilization is recorded as an upward deflection, as are decreases of optical density at 340 mμ, corresponding to DPNH oxidation. Guinea pig liver mitochondria, glutamate as substrate, K+ free isotonic medium (Expt. 466c).

and, interestingly enough, the fast respiration ceases after 26 μM $O_2$ has been utilized. The spectroscopic effects are also cyclic as with ADP. But a second addition of 400 μM calcium gives irreversible changes in optical density and in respiration rate. The latter effect is the one studied in detail by previous workers, for example see (12). Below roughly 0.5 mM the effects of calcium are reversible, and the ratio of the amounts of calcium to ADP required to give the same oxygen uptake lies between 2 and 3. If, for example, this reversible phase of the calcium effect is caused by a release of bound ADP or a breakdown of ATP, the stoichiometry of such a reaction is 2 to 3 calcium ions to one molecule of ADP or ATP, a rather reasonable figure. The irreversible effects of $Ca^{++}$ are probably caused by much more profound changes in the mitochondrial structure.

*The effect of magnesium.* — Figure 3 illustrates the increased control of respiration obtained by magnesium addition to mitochondrial suspended in a magnesium-free medium, in agreement with results from Lardy's laboratory. Succinate respiration proceeds almost without any control at all (*). Then magnesium addition

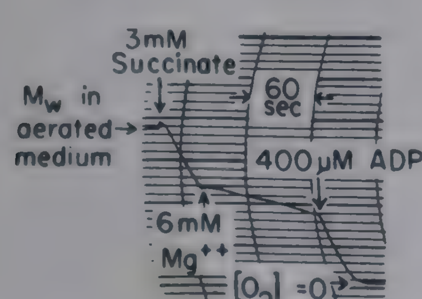


FIG. 3. — The effect of magnesium upon respiratory control. Rat liver mitochondria suspended in a medium containing 0.25 M sucrose, 5 mM versene, and 4.5 mM phosphate. The initial oxygen concentration is about 230 μM (Expt. 474b).

(*) Further experiments by H. Baltscheffsky with more tightly coupled mitochondria show that respiratory control can be retained even in the absence of added $Mg^{++}$.

nearly halts the respiration which can then be accelerated roughly 14 fold by ADP addition. Thus added magnesium restores the lost respiratory control.

*Combined effects of calcium, magnesium and ADP.* — Figure 4 illustrates first the reduction of cytochrome *b* and DPNH (recorded at 430 and 340 mμ respectively) upon addition of succinate, secondly the cycle of oxidation and reduction of cytochrome *b* and DPNH upon addition of calcium (*cf.* figure 2), thirdly the reduction of both components upon inhibiting respiration by addition of magnesium (*cf.* figure 3) and the typical cycle of oxidation and reduction caused by last addition of ADP. The 'uncoupling' agents (ADP, calcium) cause oxidations of these components, and the 'coupling' agent (magnesium) causes a reduction.

*The mechanism of respiratory control.* — In carefully prepared mitochondria there are large changes in the oxidation-reduction levels of the respiratory enzymes coincident with changes in the respiratory rate caused by changes in the level of 'coupling' or 'uncoupling' agents. These agents not only couple or uncouple oxidation and phosphorylation, but they also regulate the respiration rate. They are thus 'controlling'

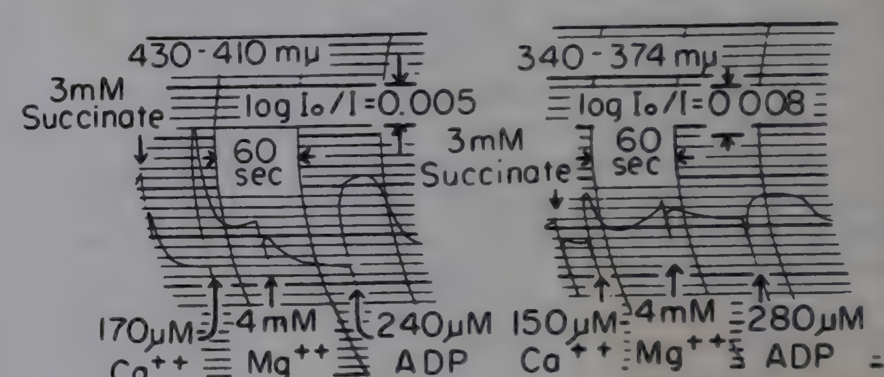


FIG. 4. — Effects of succinate, calcium, magnesium, and ADP upon the steady state oxidation-reduction levels of cytochrome *b* and DPNH. Decrease of optical densities at 430 and 340 mμ are recorded as upward deflections. Rat liver mitochondria suspended in a medium containing 0.25 M sucrose and 4.5 mM phosphate (Expt. 476c).

(magnesium) and 'decontrolling' agents (ADP, DNP, calcium, etc.). But respiratory control differs from phosphorylation efficiency. In order to account for a 10-20 fold range of control of respiration, one component of the respiratory chain must become nearly completely reduced or oxidized. We have observed DPNH to be over 99 % reduced in the controlled state (state 4) (4). That this DPNH present in the controlled state is unreactive or inhibited (as DPNH ~ I) (4) is emphasized by figure 5 in which hypotonically treated mitochondria were used : addition of a solution of DPNH gives nearly as much increase of respiration as does ADP addition, even though the mitochondria already contain DPNH (as DPNH ~ I). Thus controlling agents increase the inhibition of DPNH oxidation, and de-controlling agents do the opposite. This is the only component that need be affected to control electron transport and it may be that other components do not give the same degree of

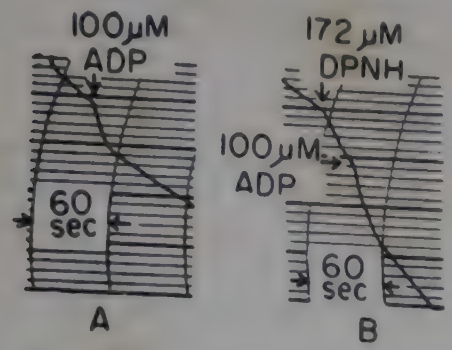


FIG. 5. — A comparison of the increases of respiration caused (A) by ADP addition, (B) by DPNH and then ADP addition. Guinea pig liver mitochondria glutamate as substrate, 0.11 osmolar medium. Oxygen utilization recorded as downward deflection (Expt. 464 A B).

respiratory control; in the inhibited state, succinate respiration is about three times as rapid as that obtained with β-hydroxybutyrate. Also no significant respiratory control has been obtained in cytochrome *c* oxidation even though phosphorylation is obtained (13. 14). Thus we can explain why respiratory control can be largely abolished even though phosphorylation efficiency is still relatively high (15, 16) on the basis that a small conversion of DPNH $\sim$ I to DPNH (*cf.* equation *1*) would diminish respiratory control without much effect on phosphorylation. According to this view, the primary effect of metabolic regulators upon the oxidative phosphorylation system is to alter the rate of electron transport without much change of P : O value. These ideas may afford a common basis for the conflicting observations of Lardy (17) and Martius (18) in the site of action of the thyroid hormone by having thyroxine affect respiratory control mostly at the DPNH stage and phosphorylation at the cytochrome *b* stage.

In the succinate system, the 'inhibited' respiration (state 4) proceeds more rapidly than when β-hydroxybutyrate is the substrate, possibly because respiratory control is obtained through inhibition of cytochrome *b* (as $b'' \sim$ I) when succinate is the substrate.

The above explanation for the mechanism of respiratory control can shed some light on the great differences in the properties of rat liver mitochondria as prepared by the methods of Lardy and Wellman (8) and rat heart muscle sarcosomes as prepared by Slater and Holton (19). There are four major differences in the preparations and all of them center about the possibility that the muscle sarcosome preparation lacks the integrity of the liver mitochondria. The first is the range of respiratory control. Liver mitochondria show a 5 to 15 fold stimulation of respiration upon addition of phosphate acceptor (16) while the heart muscle sarcosomes show a « less marked » effect (20); only one detailed experiment on this point appears to have been published by Slater and here a value of about 5 fold was recorded and no indication of the experimental range was included (15). Many data are available on the liver preparations (8, 21). The second point that is in accord with our ideas on the mechanism of respiratory control is that Holton (22) is unable to obtain any evidence at all for the presence of DPNH with an absorption maximum at 340 mµ in the muscle sarcosomes, while the liver mitochondria show up to 40 times as much DPNH as cytochrome *a* (4).

The third point, which has been adequately discussed elsewhere (16, 19), is the very low value of the P : O value obtained with succinate as a substrate for the heart muscle sarcosomes, a value of about 1.0, compared with a value 1.8 for Plaut's preparation of sarcosomes (23, 24) and close to 2.0 (21) for the liver preparation (the P : O value with succinate is 2/3 the value of 3.1 obtained with β-hydroxybutyrate (16, 20)). This discrepancy is so large that it is rather unlikely that questins of how the manometric technique should be used (19) are relevant here; in fact, Slater does not appear to have questioned the differences between his P : O values for heart sarcosomes and those of others on liver mitochondria when succinate is used as a substrate. The fourth and perhaps decisive point verifying a real difference in the Slater's heart preparations, is afforded by Slater himself (20) who quotes Holton's unpublished results on spectrophotometric studies of cytochrome *b* in heart muscle sarcosomes. Holton states that the oxidation-reduction level of cytochrome *b* is more reduced in the presence than in the absence of ADP whereas we find just the opposite results in our observations of cytochrome *b* in liver mitochondria (4). In the latter case, we have used this oxidation reaction as evidence for the participation of cytochrome *b* in oxidative phosphorylation in the liver preparation. The opposite result obtained by Holton on the muscle preparation would suggest that cytochrome *b* is not participating in oxidative phosphorylation in the muscle preparation.

In summary, four of Slater's experimental data on his own preparations distinguish them from the liver preparations; their « less marked » respiratory control, their low pyridine nucleotide content, their low P : O value, and the kinetics of cytochrome *b*. All these differences are consistent with the idea that the muscle sarcosomes studied so far by Slater have been somehow damaged in the course of their preparation. Direct evidence for damage to their respiratory control mechanism is afforded by evidence recited in Lardy's report (17) on the great range of respiratory control obtainable in an intact muscle.

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# Structural factors in metabolic regulations

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(*Received* 4 *April* 1955)

Although cytologists have long been aware that the living cell contained a number of discrete structural components and had speculated about the metabolic functions of these cell structures, it is only during the past few years that concrete biochemical data, demonstrating the association of specific enzymatic activities with individual cell structures, has been obtained. The fact that enzymes are not distributed haphazardly throughout the cell but are localised on sharply defined cell structures provides an excellent mechanism for the control of intracellular metabolism. It is this aspect of biochemical regulations that forms the basis of this paper.

Before proceeding further, it will be useful to review briefly the structural organization of the living cell. The cell is subdivided into the nucleus and the cytoplasm. The nucleus, which is generally spherical in shape, contains one or more nucleoli, chromatin material, presumably corresponding to the chromosomes seen in the cell undergoing mitosis, and the nuclear sap containing the soluble phase of the nucleus. The cytoplasm is considerably more complex. Here we can see the following cell structures under the microscope :

*(a)* variable numbers of filamentous and spherical mitochondria ;

*(b)* spherical secretory granules which can be differentiated from mitochondria by their reaction to vital stains ;

*(c)* fat droplets, which can readily be differentiated from mitochondria and secretory granules by means of their optical properties and staining reactions ; and,

*(d)* the Golgi substance, which can be seen in certain living cells as a large structure, about the size of the nucleus, characteristically located within the cell.

In addition to these visible structures, the cytoplasm also contains the basophilic substance arranged in a complex submicroscopic organization, which is now being studied intensively with the electron microscope, and a soluble phase, containing proteins, salts, etc.

How do we go about studying the biochemical functions of these cell structures ? Although several methods are currently being used to study this problem, the only one with which we will be concerned here is the so called cell fractionation technic. This is because most of our knowledge of the biochemical functions of subcellular structures have come from the application of this procedure. This is unfortunate, since, as we shall see, the cell fractionation technic is by no means perfect.

The development of cell fractionation is largely due to the work of Bensley and Claude, who early recognized that it would be necessary to isolate subcellular structures in amounts sufficient for biochemical analysis if we were to obtain accurate information about their composition and function and also make full use of current developments in biochemical knowledge. The general procedure followed in cell fractionation aims at (*a*) the preparation of tissue suspensions in such a way that the cell membranes are disrupted and the subcellular structures released, undamaged and cytologically unaltered, into a suitable medium, and (*b*) the isolation of the subcellular elements by differential centrifugation. The latter is made possible by the fact that the subcellular structures differ from each other in size as well as in density.

By this means it has been possible to isolate nuclei, nucleoli, chromatin, mitochondria, microsomes (presumably corresponding to the submicroscopic portion of the cytoplasm), the Golgi substance, and a soluble fraction from tissues. Although these subcellular elements can readily be obtained in amounts sufficient for analysis by ordinary biochemical methods, there are several limitations in the cell fractionation technic that should be considered, before the significance of the results obtained with this method can be discussed. In the first place, since most tissues used for cell fractionation are composed of more than one type of cell, the results will necessarily represent an average of the contributions of the different cell types. This is a serious limitation, since one of our objectives is to study the different tissues of the body. In some cases however this difficulty can be minimized by separating the different cell types prior to fractionation or by the correlation of quantitative cytological analysis of the tissue and the isolated material.

A more serious disadvantage introduced by the use of large populations of cells for fractionation is occasioned by the fact that all the cells of a given type within a tissue may not necessarily be the same. In the case of the parenchymal cells of the liver, for example, it is well known that the morphology of mitochondria varies considerably with the location of the cell within the liver

lobule. Since mitochondria are isolated *en masse* from all parenchymal liver cells, it is apparent that we will either have to depend upon other methods or await considerable refinements in cell fractionation before we will be able to determine whether this difference in morphology also reflects a difference in biochemical function.

Although it is possible to isolate some subcellular structures in a morphologically and cytologically unaltered form, it is important to recognize that alterations in these structures could have occurred either during cell disruption or during isolation, which were beyond our limits of detection. Among such artefacts, redistribution of materials among subcellular structures, either with or without accompanying adsorption, could occur. The question of loss of material from subcellular structures is one that has continuously plagued us, but as we shall see later, recent experiments have shown that at least some of these structures are capable of retaining very small molecules when isolated under appropriate conditions. Furthermore, adsorption, either does not occur under these circumstances or is readily recognized experimentally. Consequently, at the moment, we feel that cell fractionation can give an accurate picture of the function of subcellular structures provided that the technic is properly applied and the results are cautiously interpreted. The interested reader is referred to previous reviews (1-8) for a more detailed consideration of the problems involved in the use of cell fractionation as a cytochemical tool.

During the past five or ten years, a large amount of work has been done on the isolation of subcellular components and on the study of their enzymatic functions. I should now like to discuss the functions of various cell structures, in terms of known metabolic pathways or of the metabolism of types of compounds. Considered in this way, I think it will become obvious that metabolism must be controlled by the spatial location of enzymes and metabolites within the cell.

### *Krebs tricarboxylic acid cycle*

Much has been written about the localisation of the enzymes of the Krebs cycle in mitochondria (figure 1). The notion that the enzymes involved in this sequence of reactions were localised exclusively in mitochondria was fostered by the finding that isolated mitochondria (9) or mitochondria-containing preparations (10) would oxidize each of the acids in the cycle. On the other hand, studies (11, 12) in which the oxidation of Krebs cycle substrates by mitochondria and other cell fractions was determined, showed that although mitochondria alone were able to oxidize these compounds, their ability to do so was greatly inferior to that of the tissue as a whole. These studies showed further that the ability of the mitochondria to oxidize Krebs cycle intermediates was greatly increased by the addition of other cell fractions such as nuclei and microsomes and that when all fractions were recombined in their original proportions the oxidative capacity of the tissue was regained.

The explanation of these findings has now become clear as the result of painstaking studies on the intracellular distribution of Krebs cycle enzymes. In the

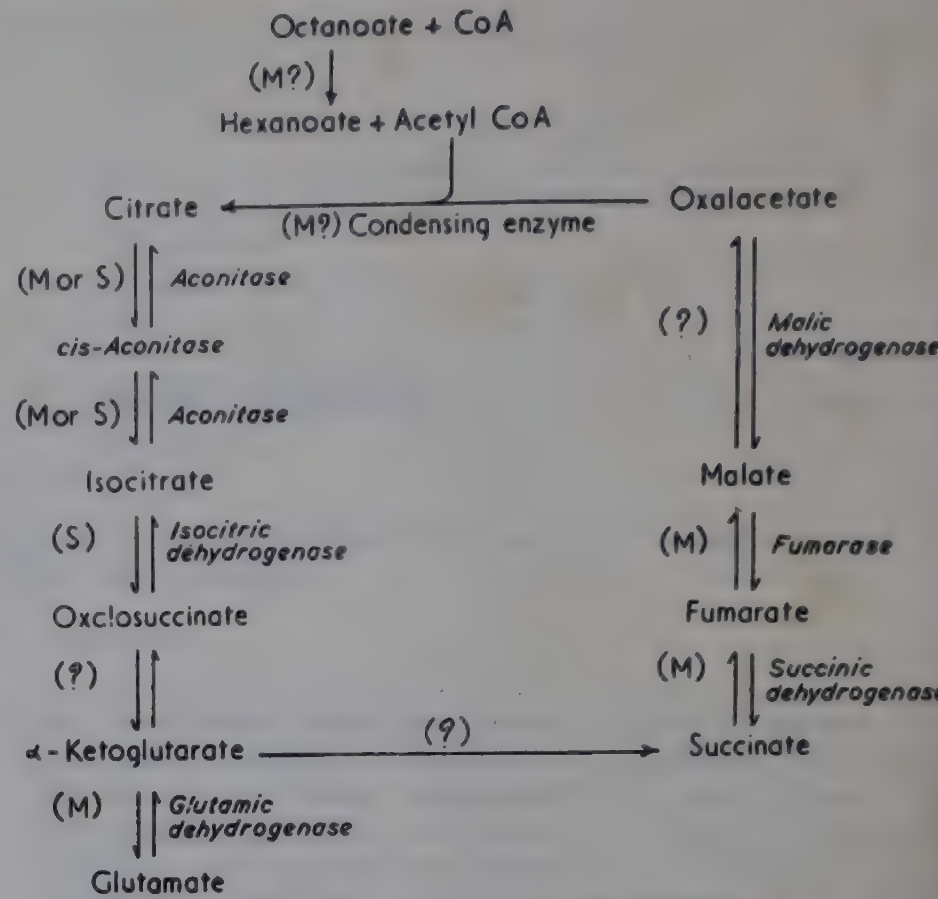


FIG. 1. — Enzymes and intermediates involved in the Krebs cycle and closely related reaction. Localisation is indicated by the symbols in parentheses as follows : (?), not known ; (M), mitochondria ; (M?), some evidence for localisation in mitochondria ; (S), soluble fraction.

first place, such studies have shown that cytochrome oxidase was localised exclusively in mitochondria (13-15). Since cytochrome oxidase is the terminal enzyme involved in the oxidation of all Krebs cycle substrates, it is immediately clear why oxygen uptake in the presence of these compounds should have been limited to the mitochondrial fraction. Other studies showed furthermore that some of the enzymes of the Krebs cycle were localised in the mitochondria while others were not. Thus, succinic dehydrogenase (13-16) and fumarase (17) were present mainly in the isolated mitochondria of liver while *iso*citric dehydrogenase (12) and aconitase (18) were recovered almost entirely in the soluble fraction of the cell. Over 80 % of the total *iso*citric dehydrogenase activity was present in the latter fraction while only 12 % was associated with the mitochondrial fraction. The small amount of activity present in the mitochondria illustrates one of the limitations of the cell fractionation technic since we are unable to decide whether this represents a real property of the mitochondria or is the result of contamination. It is our feeling, after careful consideration of the limitations of cell fractionation, that we can place reliance only on those findings in which a large amount of the total tissue enzyme activity is recovered in a single fraction and/or the concentration of the enzyme in the fraction is considerably greater than in the whole tissue. Since the *iso*citric dehydrogenase associated with mitochondria meets neither of these criteria, we are forced to conclude that oxidation of *iso*citrate must take place mainly outside the mitochondrion.

In the case of aconitase, although a similar pattern of enzyme distribution seems to exist in liver, the findings

with this enzyme raise some additional points for discussion. Dickman and Speyer (18) reported that the small amount of activity present in the mitochondria differed from the aconitase present in the soluble fraction (where most of the tissue activity was recovered) in having two pH optima, one at 5.8 and another at 7.3. The activity of the soluble aconitase was greatest at pH 7.3. Furthermore, the authors found that the mitochondrial aconitase could be converted to the soluble form by freezing and thawing the mitochondria. These findings would seem to be open to at least three interpretations : that normally the liver cell contains two types of aconitase, one soluble and the other bound to mitochondria; that aconitase is normally present in the mitochondria of the liver but is lost during their isolation; or that during the isolation of mitochondria, a small amount of aconitase was adsorbed or absorbed and its properties accordingly changed. The finding of Shepherd and Kalnitsky (19) that aconitase was localised exclusively in mitochondria isolated from rabbit cerebral cortex might be cited as evidence in favor of the second possibility, although it obviously cannot constitute proof thereof. It should be possible to test experimentally whether aconitase were being lost from or taken up by liver mitochondria, either by suitably designed isolation and washing experiments or by experiments in which isolated mitochondria were exposed to soluble aconitase.

Before terminating the discussion of the citric acid cycle enzymes, some experiments on the intracellular distribution of endogenous citric acid should be mentioned (*). These experiments were undertaken in our laboratory because the *in vitro* studies had indicated that liver mitochondria were deficient in *iso*citric dehydrogenase and aconitase. It was reasoned that if mitochondria were really deficient in these enzymes, citric acid should be present in isolated mitochondria. This proved to be the case. When liver homogenates were fractionated by differential centrifugation and the fractions analysed for citric acid, the data indicated that this compound was localised exclusively in mitochondria. Furthermore when rats were injected with fluoroacetate to block the utilization of citrate, the level of citrate in the liver was increased 7-8 times above the normal level but the citrate was still localised in the isolated mitochondria. These findings would seem to indicate that mitochondria were deficient in the enzymes utilizing citrate and also that the enzymes involved in the formation of citrate, such as the condensing enzyme, must also be localised in mitochondria.

In summary, then, it may be said that although some of the Krebs cycle enzymes appear to be localised exclusively in mitochondria, others are not. Furthermore, the distribution of several of the enzymes involved remains to be studied (figure 1). It is also clear from the results that we need to learn considerably more about the distribution of these enzymes as measured by specific one step reactions before we will really be able to determine how the Krebs cycle takes place within the cell. A study of the intracellular distribution of Krebs cycle intermediates would probably also help considerably in obtaining this information.

(*) Schneider, W. C., Striebich, M. J. and Hogeboom, G. H. unpublished.

### *Hydrogen transport*

The intracellular distribution of the enzymes and coenzymes involved in the transfer of electrons from dehydrogenases to molecular oxygen (figure 2) has been fairly thoroughly studied. It has been found, for example that di- and tri-phosphopyridine nucleotide (DPN and TPN), unfortunately measured as the mixed nucleotides, were diffusely distributed throughout the cell, with most of the total tissue nucleotides being present in the soluble fraction (20). The interpretation of these findings must await the separate determination of the intracellular

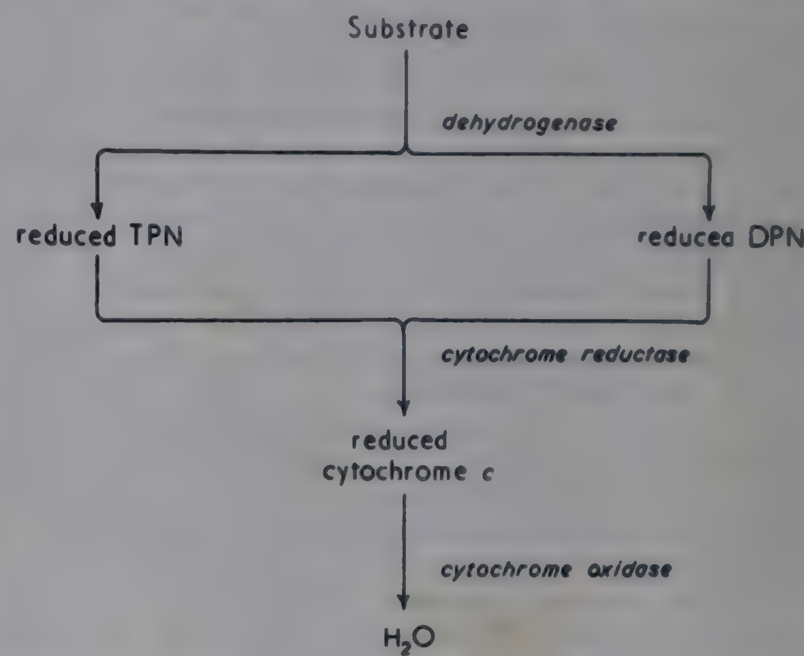


FIG. 2. — Enzymes and coenzymes involved in hydrogen transport. See text for localisation.

distribution of DPN and TPN. It would also be of considerable value to know whether the distribution of the oxidized pyridine nucleotides differs from that of the reduced pyridine nucleotides. Such determinations may not be feasible however since a considerable amount of time is consumed in the isolation of tissue fractions and a considerable alteration in the proportions of these compounds might be expected to occur during this interval.

Some of the enzymes involved in the oxidation of reduced DPN and TPN have been found to be bound to particulate material in the cell, although not localised in a single type of particle. In the case of DPN-cytochrome *c* reductase, 59 % of the total liver activity was associated with isolated microsomes and 28 % with isolated mitochondria (21), while 49 % of the TPN-cytochrome *c* reductase activity was present in mitochondria and 36 % in the microsomal fraction (12). The enzymes were concentrated to a significant extent in both fractions. Lehninger (22) has found that the oxidation of reduced DPN by isolated mitochondria, and its accompanying phosphorylation, was profoundly influenced by the physical state of the particles. If the mitochondria were distended by treatment with distilled water, the rate of oxidation was increased 3-5 fold.

This effect was opposite to that observed in studies of the activity of cytochrome *c* within mitochondria (see below) and may mean that mitochondria as isolated are relatively impermeable to reduced DPN.

Cytochrome *c* was found to have an even different distribution (23-25). The enzyme was absent from microsomes, except when these particles were isolated in media in which adsorption occurred (23, 25). Most of the cytochrome *c* of the liver (51 %) was associated with the isolated mitochondrial fraction, but a considerable amount (*ca.* 30 %) was also present in the soluble fraction. The activity of the cytochrome *c* present in isolated mitochondria was highly dependent upon the physical state of these particles. If the mitochondria were isolated in isotonic or hypertonic media (23, 24), the cytochrome *c* was highly active in the oxidation of added substrates and added cytochrome *c* had little or no influence on the rate of oxygen uptake. On the other hand, if the mitochondria were isolated or resuspended in distilled water, they were unable to catalyze these oxidations, even though they contained just as much cytochrome *c* as mitochondria in isotonic or hypertonic media. In order to obtain oxidation by water treated mitochondria, it was found necessary to add purified cytochrome *c* to the medium in a concentration which subsequent work (26) indicated was the concentration in the intact mitochondrion. These observations again illustrate the importance of osmotic factors in controlling the activity of mitochondrial enzymes. With respect to the cytochrome *c* present in the soluble fraction, no satisfactory conclusion can be reached. The experimental evidence does not permit one to conclude either that this cytochrome *c* had been lost from mitochondria or that it is normally present in the soluble portion of the cell.

The terminal enzyme of the respiratory chain, cytochrome oxidase, has been found to be localised exclusively in mitochondria (13-15). This has been shown not only by the fact that 80 % or more of the total liver cytochrome oxidase activity was recovered in the isolated mitochondrial fraction but also by more recent experiments in which it was found that cytochrome oxidase activity closely paralleled the number of mitochondria in cell fractions (27).

The fact that all of the components of the hydrogen transport sequence are not located in the same subcellular structure would at first glance seem to be an inefficient and illogical arrangement. This organisation is, however, consistent with the distribution within the cell of other enzymes. Thus the association of the cytochrome *c* reductases with microsomes seems reasonable when considered from the point of view that the soluble fraction contains dehydrogenases, pyridine nucleotides and cytochrome *c* which can react with these enzymes. The diverse localisation of these respiratory enzymes and coenzymes within the cell must mean that oxidations are considerably restricted by the existing spatial relationships.

*Oxidative phosphorylation*

One of the greatest contributions made by biochemistry in the past decade has been the demonstration that adenosinetriphosphate (ATP) is one of the key sources of energy available to the cell. Since this is the case, the mechanisms by which this compound is formed or regenerated are of greatest importance. One of the sources of cellular ATP is provided by glycolysis. The enzymes involved in this sequence of reactions will be considered later.

The largest supply of high energy phosphate is apparently produced during the oxidation of glutamate, β-hydroxybutyrate, α-ketoglutarate, *iso*citrate, succinate, and pyruvate. Each of these compounds has been shown to be oxidized by isolated liver mitochondria and to yield 2-3 moles of high energy phosphate per mole of substrate oxidized (28). These high yields of ATP apparently result from the fact that inorganic phosphate is esterified at several points in the transfer of electrons to molecular oxygen (28) since Lehninger (22) has been able to show that phosphate is esterified when reduced DPN is oxidized and more recently Nielsen and Lehninger (29, *cf.* 30-32) demonstrated that phosphorylation also occurs during the oxidation of reduced cytochrome *c*.

Our present cytochemical knowledge of oxidative phosphorylation is very vague. Although we know that mitochondria will carry out oxidative phosphorylation reactions, we do not know whether all the component enzymes involved in these reactions are localised in mitochondria. This is largely due to the complexity of the reactions involved and the difficulties in measuring them. Competive reactions, such as the hydrolysis of ATP, can interfere greatly with the measurement of the amount of phosphorylation occurring during oxidation. We know that ATP-ase is localised both in nuclei and mitochondria (13, 33), but that the enzyme is largely inactive when mitochondria are isolated under the best conditions (34). Mitochondrial ATP-ase is readily converted to an active form by relatively mild procedures, such as incubation for short periods of time at 38° C., however. Although the mechanism by which inorganic phosphate is esterified during oxidation is not entirely clear, it seems to be generally agreed that adenosinediphosphate (ADP) plays an important role in this process, perhaps as a primary phosphate acceptor, and that the enzyme, adenylate kinase, which maintains an equilibrium between the three adenosine phosphates according to the equation :

$$\text{ATP} + \text{AMP} \rightleftarrows 2\ \text{ADP}$$

functions in maintaining the ADP concentration at a constant level. According to Kielley and Kielley (34), who recovered 72 % of the total liver adenylate kinase activity in mitochondria, this enzyme would appear to be an exclusive function of mitochondria (*cf.* 35, however).

Oxidative phosphorylation is limited both by the concentration of available ADP and of inorganic phosphorus and numerous papers have dealt with how the concentration of these compounds can be maintained at levels sufficient to permit oxidative phosphorylation. This has been done *in vitro* by adding glucose and hexokinase (34) or other enzyme systems (36) to regenerate ADP from the ATP formed during phosphorylation. These methods obviously do not replenish the inorganic phosphorus supply. Within the cell other mechanisms

probably accomplish this purpose. The work of Siekevitz and Potter (37), for example, has shown that oxidative phosphorylation can be regulated within mitochondria by means of reactions that utilize ATP. The specific reaction they studied was the synthesis of citrulline. A similar purpose may also be served by reactions occurring outside the mitochondria such as glycolysis (36). The well known stimulating effect of other cell fractions on oxygen uptake by mitochondria (11, 12) is apparently due in part to the fact that these fractions can make more ADP and inorganic phosphate available. Johnson and Ackermann (38) concluded, for example, that oxidation of α-ketoglutarate by mitochondria was stimulated by nuclei by two different methods, one of which they attributed to the ATP-ase activity of the nuclei and the other to a limitation in the ability to transfer high energy phosphate to ADP. In view of the large number of other enzymatic reactions, both inside and outside the mitochondria that require ATP, it is evident that a number of such regulatory systems could exist.

One final method by which oxidative phosphorylation could be regulated in mitochondria within the cell should be mentioned. This method is suggested by the finding (22, 29) that the phosphorylations accompanying the oxidation of externally added reduced DPN and cytochrome *c* were considerably increased by increasing the permeability of mitochondria.

### *Anaerobic glycolysis*

As we all know, the enzymes involved in the Embden-Meyerhof glycolytic sequence of reactions (figure 3) were all studied originally in aqueous extracts of tissues. It is consequently not surprising perhaps that enzymes such as hexokinase (39), phosphorylase (39), phosphoglucomutase (39), aldolase (9), and lactic dehydrogenase (*) should have been recovered almost entirely in the soluble fraction of tissues obtained in cell fractionation experiments.

The work of LePage and Schneider (40), however, in which the anaerobic formation of lactic acid from glucose was studied, showed that the soluble fraction of the tissue was able to catalyze these reactions only about one half as rapidly as the unfractionated tissue. Furthermore, they found that although the other cell fractions were essentially unable to promote glycolysis by themselves, when added to the soluble fraction in their original concentrations, the glycolytic activity of the original tissue was regained. These findings consequently indicated that the activity of the soluble fraction was limited by accessory factors present in the particulate components of the cell. Although the nature of these factors remains to be determined it is clear that glycolysis, just as the Krebs cycle, is limited by the spatial localisation of its constituent enzymes.

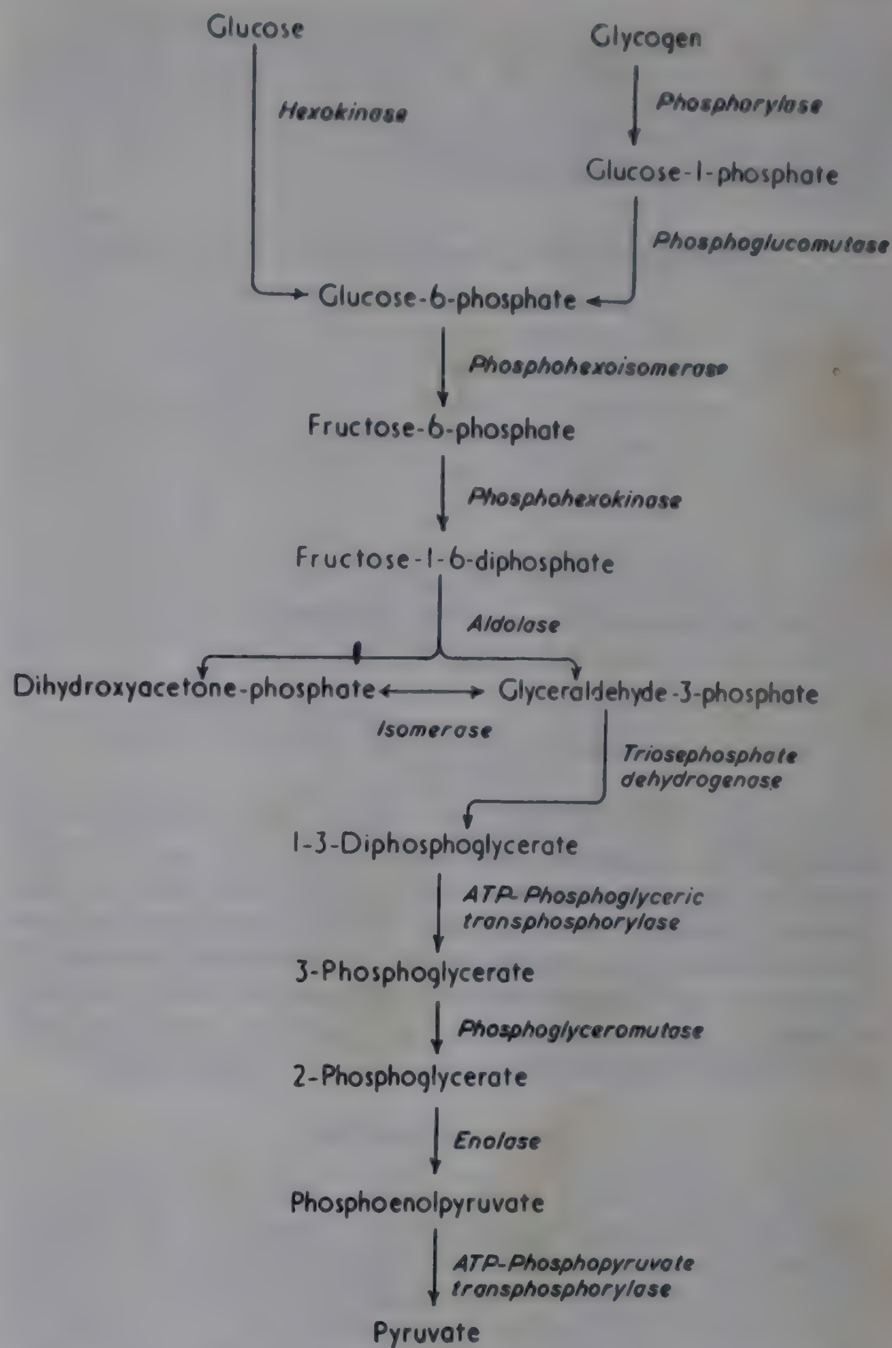


FIG. 3. — Enzymes and intermediates in glycolysis.

### *Urea cycle*

Although the enzymatic steps involved in the formation of urea (figure 4) have not been completely elucidated, the information so far available indicates that the enzymes involved in this sequence of reactions are not all located in the same part of the cell. The only enzyme that has been studied by means of cell fractionation is arginase, and the results obtained with it have not been entirely satisfying. The studies of Schein and Young (41) and Ludewig and Chanutin (42), although done somewhat differently, both showed that this enzyme was present in all cellular fractions, but was concentrated only in the nuclear fraction. On the other hand, arginase was present in much lower concentrations in nuclei isolated in non-aqueous media (43). These discrepancies and the diffuse distribution of arginase observed in aqueous media indicate that this enzyme requires further investigation.

The synthesis of citrulline from ornithine has been detected in isolated liver mitochondria (44) and has been found to be highly dependent upon simultaneous oxidative phosphorylation (45). Presumably the synthesis of citrulline by this mechanism is similarly limited in the intact cell. In suitable soluble tissue preparations, however, the synthesis of citrulline occurs anaerobically in the presence of ATP (46) and has been resolved into

(*) Hogeboom, G. H. and Schneider, W. C. unpublished.

3 reactions (figure 4) : the formation of carbamylglutamate, the conversion of the latter to an unknown intermediate, compound X, and the condensation of this intermediate with ornithine. These reactions have not been studied cytochemically.

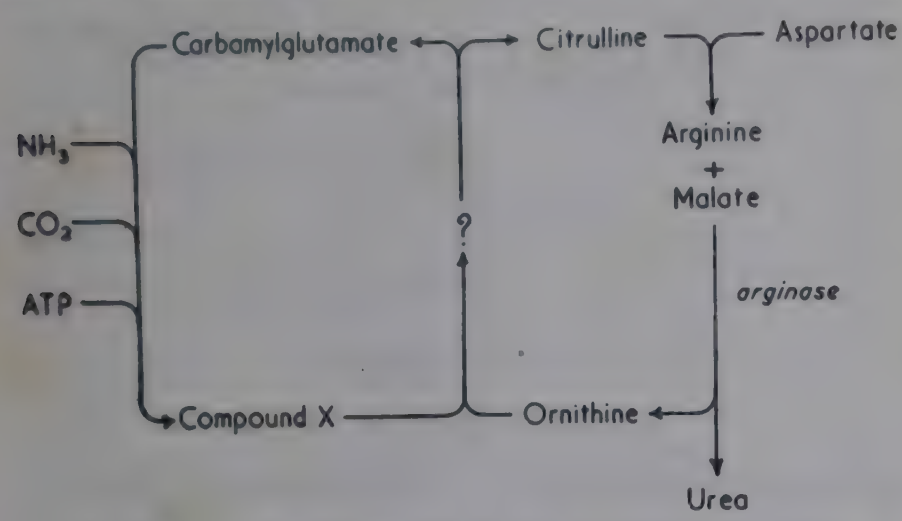


**Fig. 4.** — Intermediates in the formation of urea.

The final link in the urea cycle, the conversion of citrulline to arginine, has been shown to require both soluble and particulate-bound enzymes (47). The soluble fraction was believed to contain the enzyme required for the synthesis while the insoluble fraction of the tissue was thought to contribute the necessary supply of energy. The nature of the enzymes involved and their intracellular distribution remains to be determined.

### *Lipid metabolism*

The oxidation of fatty acids, such as caprylic or octanoic acid, has been shown to be localised exclusively in isolated mitochondria (9, 48). Oxidation of these compounds apparently proceeds in a stepwise fashion involving activation, by the formation of a coenzyme A derivative, oxidation, hydration, and reoxidation, followed by cleavage to yield acetyl-CoA and a fatty acid shortened by two carbon atoms (49, 50). The oxidation of the latter can then continue in a similar fashion until complete oxidation has occurred. The acetyl CoA formed can be condensed with oxalacetate and metabolized through the citric acid cycle (figure 1) or used for other purposes. Each of the enzymes involved in the oxidation of fatty acids has been isolated from beef liver mitochondria and purified (50). Although this finding would suggest that these enzymes are localised in mitochondria, it cannot be accepted as proof. In support of the supposition that at least some of the fatty acid oxidizing enzymes are localized in mitochondria is the finding that these granules contain 52 % of the total liver CoA (50*a*). Since this represents a minimum value due to incomplete recovery of mitochondria during isolation and purification, it would appear that many of the enzymatic reactions in which this compound functions must probably be localised in mitochondria.

The biosynthesis of fatty acids has been postulated to take place by reversal of the fatty acid oxidation sequence (49). In the early experiments of Brady and Gurin (51) acetyl-CoA was considerably less effective than acetate as a precursor of tissue fatty acids. These workers measured the incorporation of labeled acetate into the fatty acids of their tissue preparations and found that both a mitochondrial fraction and a particle free soluble fraction were necessary for incorporation to occur. They were able to solubilize the factors present in mitochondria by treating them with acetone and extracting with buffers. In more recent experiments, however, a requirement for CoA, ATP and DPN was demonstrated by pretreatment of the enzyme preparations with charcoal (52).

The synthesis of tissue cholesterol from acetate appears to be localised somewhat differently in the liver cell from the synthesis of fatty acid. Bucher *et al.* (53) reported that both the microsomal and soluble fractions were necessary for cholesterol formation and in more recent work (54) demonstrated that only the larger microsomes were active. Rabinowitz and Gurin (55), on the other hand, reported that the synthesis of cholesterol from acetate occurred in a system consisting of mitochondria, soluble fraction, ATP and DPN. Their mitochondrial fraction was obtained at such high speed, however, that it probably contained a large part of the submicroscopic material of the liver homogenate as well.

The hydroxylation of steroids by beef adrenal tissue fractions has received considerable attention during the past few years. The observations of Hayano and Dorfman (56) showed that an insoluble tissue fraction sedimentable at low speed was capable of hydroxylating desoxycorticosterone at carbon 11 to yield corticosterone. Sweat (57) independently found that the reaction was catalyzed only by the isolated mitochondrial fraction. More recently, Brownie and Grant (58) showed that isolated adrenal mitochondria catalyzed the hydroxylation reaction and reported that simultaneous oxidative phosphorylation was necessary to maintain the reaction. In more purified preparations of the enzyme made from acetone powders, Hayano and Dorfman (59), on the other hand, observed that fumarate, TPN and oxygen were the only necessary cofactors. Similar requirements in the metabolism of estradiol by rat liver have been found necessary by Riegel and Mueller (60). The nature of the estradiol oxidation products has not yet been determined beyond the finding that both water soluble and protein bound metabolites were produced. The oxidation of progesterone by adrenal preparations has been studied by Plager and Samuels (61), who reported that oxidation was catalyzed by a supernatant fraction (judged to be reasonably free of particulate matter on the basis of their centrifugal conditions) in the presence of added ATP and DPN. The oxidation proceeded *via* two pathways :

progesterone ⟶ desoxycorticosterone

progesterone ⟶ 17-hydroxyprogesterone
→ 17-hydroxy-11-desoxycorticosterone (Compound *S*)

Desoxycorticosterone was not formed from Compound *S*.

Studies on the synthesis of phosphatides have been initiated by Kennedy and by Kornberg and Pricer. Kennedy (62) first observed that inorganic phosphate was rapidly incorporated into these compounds by

isolated mitochondria. The incorporation was dependent upon oxidative phosphorylation and was stimulated by an unfractionated supernatant containing microsomes and the soluble components of liver. He also found that choline was incorporated into the lecithin of mitochondria by two pathways. In the first (63), phosphorylcholine did not play an intermediate role but the presence of both ATP and CoA was necessary. In the second mechanism, which involved phosphorylcholine (64), phosphorylcholine was first transformed to cytidinediphosphate choline in the presence of cytidine triphosphate and then to lecithin (65).

One of the earliest observations made with the cell fractionation technic was the finding that an esterase, measured by hydrolysis of methyl butyrate, was localised in microsomes (66). This observation is of especial interest in view of recent studies on esterified cholesterol and vitamin A. Ganguly and Deuel (67) observed that the enzyme hydrolyzing vitamin A ester was localised in the microsome fraction of liver while Schotz *et al.* (68) found that the enzyme hydrolyzing cholesterol acetate was similarly distributed. The substrates for these enzymes were localised in entirely different portions of liver cell, however. Thus esterified vitamin A, which accounts for over 95 % of the total liver vitamin A content, was localised almost exclusively in the fatty fraction which rises to the top of the centrifuge tube during centrifugation (69). The esterified cholesterol of liver was also recovered mainly in this fraction (70). On the other hand, free cholesterol was localised almost entirely in microsomes (70) while vitamin A alcohol was distributed more diffusely in the cell (69).

The oxidation of choline and of betaine aldehyde by cell fractions has been studied by a number of workers (71-77) and is still the subject of considerable controversy due to the lack of agreement on the essential cofactors involved in these oxidations and on the correct methods of measurement. The initial observation that choline dehydrogenase was localised in mitochondria (71, 72) has not been challenged but the contention that betaine aldehyde dehydrogenase was a function of mitochondria (73) has been disputed by others who claim that this enzyme was localised in the soluble fraction (74, 75).

It is apparent from this discussion of lipid metabolism that many of the enzymes involved have only been detected in various cell fractions. Furthermore, until the mechanisms involved in the action of these enzymes can be clarified and the individual enzymes involved can be measured separately, it will not be possible to obtain an accurate picture of the intracellular localisation of lipid metabolism.

### *Metabolism of nucleic acids and rela ed compounds*

A number of studies have been made on the intracellular distribution of enzymes concerned with the metabolism of nucleic acids or related compounds. The enzymes involved in the depolymerization of ribose and deoxyribose nucleic acids (RNA and DNA) have been found to be localised mainly in mitochondria (78-80). This finding was rather surprising since DNA had been found to be localised in the nucleus while RNA, although present in all parts of the cell, was concentrated mainly in the microsomes. It was suggested (78) that since these enzymes were localised in the mitochondria and thus in close proximity to a source of energy that they might perhaps function in nucleic acid synthesis.

The results of *in vivo* studies on incorporation of labelled compounds (81-85) argues against the mitochondria being the site of RNA synthesis. In these experiments, it was found that nuclear RNA was renewed much more rapidly than the RNA in any cytoplasmic fraction and it was consequently suggested that this nucleic acid may be synthesized in the nucleus, although the alternate possibility that nuclear and cytoplasmic RNA are synthesized independently at different rates could not be ruled out.

On the other hand, recent studies have shown that certain nucleic acid precursors are formed in isolated mitochondria. The pyrimidine, orotic acid, which is extensively used in nucleic acid synthesis, appears to be synthesized exclusively in mitochondria (86) (*) according to the following reaction sequence :

$$NH_3 + CO_2 + \text{aspartate} \xrightarrow[\text{ATP}]{\text{glutamate}} \text{ureidosuccinate} \rightarrow \text{orotic acid.}$$

The synthesis of certain nucleotide coenzymes by a reaction mechanism that might be thought to be involved in nucleic acid synthesis has been studied in liver cell fractions. Synthesis of DPN according to the equation :

$$\text{nicotinamide mononucleotide} + \text{ATP} \rightarrow \text{DPN} + \text{pyrophosphate,}$$

has been found to be localised exclusively in the nucleus of the liver cell (87). Over 90 % of the total tissue activity of this enzyme was recovered in the isolated nuclei. Studies on the pyrophosphorolysis of the uridine coenzymes, uridine diphosphate glucose (UDPG) and uridine diphosphate acetylglucoseamine (UDPAG) (88), by reactions analogous to the following :

$$\text{UDPG} + \text{pyrophosphate} \rightarrow \text{UTP} + \text{glucose-1-phosphate,}$$

have shown that these enzymatic reactions occur in the rat liver nucleus as well. These findings suggest that the synthesis of UDPG and UDPAG by reversal of these reactions may also take place in the nucleus.

On the other hand, the synthesis of flavine adenine dinucleotide (FAD) according to the equation :

$$\text{flavin mononucleotide} + \text{ATP} \rightarrow \text{FAD} + \text{pyrophosphate,}$$

has been found to be localised exclusively in the soluble fraction of liver homogenates. It should be mentioned that the localisation of the FAD and DPN synthesizing enzymes is considerably different from that of FAD and DPN. About 65 % of the total liver FAD was found to be localised in mitochondria (**) while DPN, on the other hand, was recovered mainly in the soluble fraction of the liver (20).

Other enzymes involved in nucleic acid metabolism that have been studied include ATP and AMP (adenylic acid) phosphatases, adenosine deaminase, nucleoside

(*) Lagerkvist, V. unpublished, quoted by Reichard (86).
(**) Schneider, W. C. and Hogeboom, G. H. unpublished.

phosphorylase and uricase. Two types of ATPase appear to exist in the liver cell. The one, activated by calcium ions, is present in the nucleus, while the other, activated by magnesium, is localised in the mitochondria (35). Dephosphorylation of AMP appears to be concentrated in nuclei and the small submicroscopic particles (89). Adenosine deaminase and nucleoside phosphorylase, which catalyses the reaction :

nucleoside + inorganic phosphate ⟶ ribose or desoxyribose phosphate + purine or pyrimidine,

have been recovered almost exclusively in the soluble fraction of liver (90). In other tissues, however, studies have shown that these enzymes may be localised in the nucleus (43). The latter experiments are open to question, however, because only the whole tissue and the isolated nuclei were assayed for enzymatic activity. The validity of the enzymatic assay was consequently not established

The enzyme uricase was at first (90, 91) recovered mainly in the mitochondria isolated from liver homogenates. Subsequent studies showed, however, that most of the uricase activity could be separated from the bulk of the isolated mitochondria, in a fraction containing numerous small dense granules and accounting for only a small fraction of the original mitochondria (89, 92). It is not at present clear whether these granules are mitochondria or not.

### *Proteins and amino acids*

Very little is known, from the cytochemical standpoint, of the metabolism of proteins and amino acids. Maver and Greco (93) studied the distribution of the proteins degrading enzymes in liver. They found that hemoglobin was hydrolyzed by both the nuclear fraction and the mitochondrial fraction, with the latter having the greater activity. In subsequent work (94), in which nuclei were prepared in a much higher state of purity by procedures designed to eliminate mitochondria and whole cells, it was found that the nuclei had very low activity. It can be concluded that in liver, proteolytic activity is probably confined to mitochondria.

A number of enzymes concerned with the metabolism of amino acids also appear to be localised in mitochondria. Glutaminase I, (95), which catalyzes the desamidation of glutamine in the presence of inorganic phosphate, appears to be associated exclusively with mitochondria. On the other hand, glutaminase II, which desamidates glutamine in the presence of pyruvate or other keto acids, is found in the soluble fraction of the tissue (96). The oxidation of tyramine has also been found to be localised in mitochondria (97, 98), and according to a preliminary report of Hird and Rowsell (99), the transaminase catalyzing the reaction :

glutamate + phenylpyruvate → α-ketoglutarate + phenylalanine,

is similarly localised in the cell. On the other hand, only a small amount of the glutamic-oxalacetic transaminase is present in isolated mitochondria (100) and the majority of the activity of this enzyme in liver appears to be present in the soluble portion of the cell (99).

Glutamic dehydrogenase, which catalyzes the oxidative deamination of glutamate to α-ketoglutarate, and is thus closely related to the Krebs cycle (figure 1), has been found to be localised exclusively in mitochondria (101). Over 85 % of the total liver activity of this enzyme was recovered in the isolated granules. The activity of the enzyme was profoundly influenced by the physical state of the mitochondria. Suspension of the mitochondria in distilled water, for example, increased the activity of the enzyme several fold. In order to measure the full glutamic dehydrogenase activity of the mitochondria it was necessary to disrupt their membranes completely.

A number of studies have been concerned with the incorporation of amino acids into tissue proteins. Early experiments showed that the amino acids were incorporated into microsomal proteins more rapidly than into the proteins of other cellular fractions and formed the basis for studies of the incorporation of amino acids into proteins in homogenate systems *in vitro*. In the experiments of Siekevitz (102), it was observed that incorporation occurred when mitochondria and microsomes were incubated together, especially if conditions permitting rapid oxidative phosphorylation by the mitochondria were established. He found furthermore that if the mitochondria were incubated separately with an oxidizable substrate and cofactors, a soluble factor was produced which enabled the microsomes to incorporate alanine in the absence of mitochondria. More recently Zamecnik and Keller (103) have found that the requirement for mitochondria could be eliminated by using small amounts of the soluble fraction of the cell supplemented with cofactors permitting glycolysis under anaerobic conditions. If the incorporation of amino acids in these experiments can really be accepted as representing protein formation, it would seem that the ability to perform this function resides in the microsomes but that it is controlled by the availability of energy produced either by anaerobic glycolysis or by oxidative phosphorylation.

The synthesis of peptide like bonds in cell free liver suspensions was first accomplished by Cohen and McGilvery (103). They observed that the synthesis of *p*-aminohippuric acid was catalyzed by the insoluble residue of tissue homogenates under conditions in which oxidative phosphorylation occurred. Kielley and Schneider (105) subsequently demonstrated that the synthesis of this compound was localised in isolated mitochondria, as shown by the recovery of 90 % of the total tissue activity in these granules. The synthesis of the closely related hippuric acid has also been detected in isolated mitochondria (106).

### *Intramitochondrial regulations*

The foregoing discussion has been directed toward considering how metabolism of various compounds was limited or controlled by the spatial localisation within the cell of the enzymes and coenzymes involved. To this, we must add a still finer distinction : control of metabolism by structural factors within particulate structures.

The evidence that we have on this point deals entirely with mitochondria. Recent work with the electron microscope has demonstrated that mitochondria possess a characteristic substructure consisting of a thin, double walled surface membrane and of numerous parallel double walled lamellae (107, 108), beginning at or near the membrane and extending almost entirely across the body of the mitochondrion.

Physical chemical studies of isolated mitochondria have shown that when their membranes are completely ruptured, more than half of the total mitochondrial mass appears in the form of soluble proteins of characteristic sedimentation behaviour while the remainder appears as submicroscopic particles (109, 110). About half of the total nitrogen content of the mitochondria is also solubilized when these granules are distended by suspension in distilled water (*). The submicroscopic particles would appear to be remnants of the membrane and the internal lamellae while the soluble material probably represents the phase existing between the mitochondrial lamellae.

The enzymatic properties of the soluble and particulate fractions of the mitochondria have been found to be quite different. Thus the soluble fraction contains almost the entire mitochondrial content of enzymes such as glutamic dehydrogenase (101), fumarase (17), ribonuclease and desoxyribonuclease (78), while cytochrome oxidase, cytochrome *c*, DPN-cytochrome *c* reductase, and succinic dehydrogenase (111) remain bound to particulate material.

On the basis of these findings, a highly complex and highly organized arrangement of enzymes within mitochondria can readily be visualised.

### *Concluding remarks*

During the course of this presentation, it has not been possible to consider either all of the enzymes that have been studied with the cell fractionation technic or the large number of studies that have dealt with plant tissues or animal tissues other than liver. Perhaps these findings will be considered in the discussion to follow.

Several major conclusions can be reached from the results that have been reviewed. We have seen that enzymes are sharply localised within the cell rather than randomly distributed throughout the cell. Consequently metabolism must be restricted to a considerable degree by the intracellular location of the enzymes involved, since any enzyme in a metabolic sequence is strictly dependent upon other enzymes for a supply of its substrate. Although our present knowledge is still obviously very incomplete, it nevertheless seems clear that all of the enzymes needed to complete a given sequence of reactions, as for example those of the Krebs cycle, are not necessarily localised within the same subcellular structure or even in the same part of a subcellular structure. Thus all parts of the cell must be mutually dependent to a greater or lesser degree.

(*) Schneider, W. C., Striebich, M. J. and Hogeboom, G. H. unpublished.

Another conclusion that can be reached from the studies here reviewed is that physical factors, other than intracellular location, may play an important role in metabolic regulation. The marked changes in the metabolism of mitochondria that have been observed *in vitro* under conditions in which the permeability of their surface membranes was increased serve as an example of such factors. Since nuclei also possess a surface membrane and submicroscopic membranous structures appear to be present in the Golgi substance and in the cytoplasm, permeability and its regulation by factors such as pH and tonicity may be of great importance in controlling metabolism within the cell.

The fact that a large proportion of the enzymatic functions of the cell that have so far been studied have been found to be localised in the mitochondria should in no way lead to the conclusion that other cellular structures are metabolically inert. On the contrary, it would seem logical to expect that as more and more enzymes are discovered and studied, other cell structures will assume greater importances as sites of enzymatic functions. The fact that the only functions localised exclusively in the nucleus so far have been synthetic ones is perhaps indicative of the type of enzyme that can be expected to be found localised in the nucleus, Golgi material, etc., in future work. Unfortunately, very few synthetic reactions can at present be studied adequately because their reaction mechanisms are not clearly understood. Until these mechanisms are clearly defined, we will necessarily be limited to measurements of the overall reaction, and, as we have seen from measurements of oxygen uptake with Krebs cycle substrates, the localisation of enzymes by such determinations can be quite different from that obtained when the individual enzymes concerned are studied. Cytochemical results must consequently be repeatedly re-evaluated and reappraised as new advances are made.

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# Microscopy and differential centrifugation

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(*Received* 19 *September* 1955)

The microscope is today better able to help interpret the data obtained by differential centrifugation, for there are now available two techniques of great power : (*a*) electron microscopy of ultrathin sections and (*b*) histochemical staining methods for enzymes.

## ELECTRON MICROSCOPY

In but a few months, the study of ultrathin sections of isolated fractions by electron microscopy has demonstrated its importance in this field (1-3). Indeed, it seems that questions such as the existence of diverse types of mitochondria and microsomes can no longer be approached without the use of the electron microscope.

Improved methods of fixation, embedding and sectioning have led to the description of the fine structure of mitochondria (4-8), of membranes called endoplasmic reticulum (9-11), small dense particles associated with the reticulum (12), and other intracellular structures (13, 8). These methods and descriptions provide, for the first time, a reliable means of identifying individual particles in fractions isolated by differential centrifugation.

We wish to report briefly our use of electron microscopy for two problems : (*a*) The search for an isolation medium in which the elution of materials from mitochondria is at a minimum, and (*b*) the evaluation of the evidence adduced by Laird *et al.* (14) for the existence of a new class of mitochondria, rich in ribonucleic acid as well as succinoxidase.

*Polyvinylpyrollidone-sucrose as an isolation medium*

The medium we have used is a modification of those suggested by Woods (15), and Greenfield and Price (16).

We have found that the shape of rat liver mitochondria (seen by phase-contrast microscopy) and the fine structure (seen by electron microscopy) are best preserved by final concentrations of polyvinylpyrollidone (PVP), 7.3 g./100 ml., and 0.25 M sucrose, at an initial pH of 7.6-7.8. The pH of the resulting homogenate is between 6.9 and 7.1.

Mitochondria isolated from this medium are generally elongated, essentially as from 0.88 M sucrose. The PVP-sucrose mitochondria maintain their elongate form for much longer periods of time than do hypertonic sucrose mitochondria, when they are examined under a coverslip. The hypertonic sucrose mitochondria fairly quickly become much enlarged spheres, each with a clear interior and a dark crescent-shaped area at one pole. Although the PVP-sucrose mitochondria will round out too, they do so much more slowly and remain dark ; they do not enlarge into the clear crescent-containing spheres.

The fine structure is beautifully preserved in mitochondria isolated from PVP-sucrose. The uniform density of such mitochondria suggests that they have suffered little or no loss of material during isolation. The outer membranes and the *cristae mitochondriales* are

very distinct. Most mitochondria appear considerably better preserved than those isolated from 0.44 M sucrose-citrate homogenates by Witter *et al.* (2).

The fixative we have used consists of osmium tetroxide, 2 g./100 ml., PVP, 7.3 g./100 ml., and 0.25 M sucrose, the mixture brought to neutrality with alkali. In each of three experiments, we found the fine structure to be best preserved at pH 7.0. Even at pH 6.5 and pH 7.5 many *cristae* areas were markedly swollen, forming irregular vesicles. In contrast, the microsomal material was still well preserved.

Like the morphologic integrity, the biochemical integrity of the mitochondria is well preserved when isolated from PVP-sucrose homogenates. This is indicated by the 'ATPase latency' of the fresh mitochondria and their capacity for oxidative phosphorylation.

Measured in the system of Lardy and Wellman (17), the PVP-sucrose mitochondria show low 'ATPase' activities when fresh, and activities 6-11 times higher in the presence of dinitrophenol or after ageing for 20 minutes at 37° C. The fresh 0.88 M sucrose mitochondria have considerably higher activities, so that there is much less activation by DNP or ageing.

The increases in 'ATPase' activity with DNP or ageing are of the same order of magnitude with mitochondria prepared in PVP-sucrose and in 0.25 M sucrose.

The PVP-sucrose mitochondria are like those from isotonic sucrose in their capacity for oxidative phosphorylation. Using α-ketoglutarate as substrate, in the presence of adenosine-5′-monophosphate, glucose, hexokinase, cytochrome *c* and DPN, but in the absence of fluoride, the P:O ratio was 2.8 for 0.25 M sucrose mitochondria and 2.7 for PVP-sucrose mitochondria.

The electron migrographs, along with the ATPase and oxidative phosphorylation data, suggest the possibility that Greenfield and Price's finding (16) regarding the distribution of catalase in fractions obtained from PVP-sucrose is not unique, and that mitochondria isolated from this medium might retain their original enzyme complement during isolation better than those from sucrose. We are currently testing the distribution of several enzymes among the fractions isolated from both sucrose and PVP-sucrose homogenates : dehydrogenases like *iso*citric dehydrogenase and phosphatases like inorganic pyrophosphatase, which from the sucrose data appear to be concentrated in the 'soluble fraction' and virtually absent from the mitochondria.

### *Is there a class of mitochondria rich in ribonucleic acid ?*

Laird *et al.* (14) separated from the so-called fluffy layer (above the sedimented mitochondria pellet) a fraction with high content of both ribonucleic acid and succinoxidase. We have suggested earlier (18) that in the presence of microsomal contamination one could not safely attribute the RNA of the fraction to the mitochondria. We used phase contrast microscopy to detect the microsomes, an admittedly shaky method for particles of such small dimensions. Since then, other investigators (19, 20) have presented chemical evidence for the presence of microsomes. On the other hand, most recently chemical data has been reported (21) to support Laird *et al.*'s viewpoint. Yet it is in the nature of such chemical data that it can not be direct. Morphologic evidence can be.

The small mitochondria to which they attributed both ribonucleic acid and succinoxidase were observed by Laird *et al.* with phase contrast and electron microscopy. However, this was before the time of ultrathin sectioning. Instead, a drop of fixed fraction was simply dried on the formvar film, shadowed and photographed. They concluded, « the electron micrographs... indicate that this material consists almost exclusively of one type of particle and that the PNA of this material is not due to freely suspended microsomes ».

Our sections show the presence of many small mitochondria, about one micron or less in length, with typical fine structure. The sections also show a great many 'ballooned' mitochondria, spheres containing crescent-shaped areas in which are situated the *cristae mitochondriales*. These *cristae* are apparently in various stages of disintegration. In addition, there are empty spheres and other particles whose nature has not yet been determined.

Most important for our present discussion is the abundance of microsomal material. This consists of typical endoplasmic reticulum membranes with attached dense granules.

A fuller description of this fraction will be presented elsewhere, together with : (*a*) our biochemical data on the fraction (*b*) a consideration of supporting data presented for their view by Laird *et al.*, and (*c*) reasons for our feeling that the fluffy layer may be, in part at least, an artifact, resulting from damage to mitochondria during homogenization and washings.

## HISTOCHEMICAL STAINING METHODS

These methods have not thus far been successfully applied to the study of isolated fractions. However, they have revealed enzyme distribution among the cells of tissue sections which must be taken into account when intrepreting data from differential centrifugation.

It is well recognized that the enzyme activity of a given fraction obtained by differential centrifugation is an average figure. Even if it were possible to free the mitochondrial fraction, for example, of all contamination by other cell particulates, enzymic differences existing among diverse mitochondria within a cell and among different cell types would still be merged into the average. This limitation is particularly troublesome when the organism is subjected to altered conditions which may shift the relative proportion of different mitochondria within a cell or of different cell types in the organ.

On the other hand, histochemical staining methods do not, in principle, suffer from this limitation. Sections of fixed or frozen tissue are stained to visualize the sites of enzyme activity and then the individual cells are viewed directly, with the microscope (22).

Although the biochemist often thinks of the liver parenchyma as a uniform population of cells, marked differences in enzymic properties have been demonstrated among these cells by staining methods. Thus in rat liver, the cells near the central veins have higher esterase activity than those near the portal areas (23), and lower activity of alkaline phosphatase (24) and of the electron

transport enzyme (22) demonstrated by the tetrazolium technique with succinate as substrate (25). Such differences are even more striking in other organs. In rat kidney, for example, the proximal tubules are extremely rich in alkaline phosphatase (26); the electron transport enzyme (22) with succinate as substrate is negative in the glomeruli and highly active in the proximal convolutions and the ascending limbs of Henle's loops (27).

Earlier biochemical analyses showed the liver tumors of rats fed 3-dimethylaminoazobenzene to be rich in alkaline phosphatase. Subsequent histochemical staining methods showed that this activity is almost completely localized in the stroma and necrotic tissue, and that the tumor cells themselves are not rich in this enzyme (28, 29).

These staining methods can often reveal enzyme activity in single cells of a tissue, cells which would be lost completely in the average activity of differential centrifugation. Thus, an individual sinusoidal cell rich in alkaline phosphatase can readily be detected in a microscope field of rat liver stained by the azo dye technique for the enzyme (26). Stained sections show these alkaline phosphatase-rich sinusoidal cells to increase in number following partial hepatectomy. They also reveal an increase in the activity of the bile canaliculi following such surgery. We have elsewhere (26) noted the usefulness of such sections in helping avoid errors in interpretation of biochemical data for the nuclear fraction obtained from regenerating liver by differential centrifugation.

We have also discussed elsewhere (22, 24) the present limitations of staining methods on an intracellular level. But increasing progress in overcoming these difficulties can be confidently predicted. With such progress will come the fuller realization of the potentialities of these methods.

*Aknowledgements*

G. E. Palade and M. L. Watson, of the Rockefeller Institute for Medical Research and L. Ornstein, of the Mt. Sinai Hospital of New-York, have given invaluable guidance in the electron microscope work. M. L. Watson prepared the electron micrographs of the PVP-sucrose mitochondria, and L. Ornstein generously made available the facilities of his electron microscope laboratory.

It is also a pleasure to acknowledge the collaboration in the biochemical work of A. Silver, F. Burnett, and M. Glickman.

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# Hormonal regulatory mechanisms

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(*Received* 20 *June* 1955)

## INTRODUCTION

Although hormonal effects and actions have been studied for more than half a century, the precise mechanism of action of any hormone has not yet been clearly established. There have been numerous reports describing the action of certain hormones on enzymes, cell extracts or on cellular particles, but in no case has the described effect been generally accepted as a complete explanation for the mechanism of action of that hormone. In some cases the effect described has not been clearly localized as to exact site of action, while in other cases it has not been clear that the observed hormonal effect was necessarily related to the physiological effects noted in intact animals. These remarks are not intended to reflect a feeling of pessimism, but to indicate the general difficulties which have been encountered in the analysis of hormone action. Two outstanding difficulties will be considered briefly and are (*a*) establishement of reproducible hormonal effects in a definite soluble system, and (*b*) extrapolation of *in vitro* effects to the physiological action of the hormone in the intact animal.

If we examine the first problem more carefully, we are tempted to feel that some hormones act at a level of organization more complex than that existing in a soluble homogeneous system. At present it seems that hormones act on specialized colonies of cells and the action is generally lost if the cell structure is disrupted., Basic enzymatic reactions proceed in mammals without the presence of the hormones and the mammalian hormones do not influence the metabolism of unicellular organisms. Moreover, the hormones act in tremendous dilutions and very small amounts suffice to produce marked changes in intact animals. The possibility exists that some of the hormones exert their action on highly organized groups of molecules.

On the other hand, there is reason to feel that the hormones may act at the molecular level. For example, recalling the elucidation of the mechanism of the biosynthesis of urea, we find a period when urea synthesis could be studied only in the whole animal; later its formation was studied in perfused livers, then in liver slices, and was finally obtained in extracts of cells. By analogy, it seems that a systematic analysis of hormone action might also proceed from studies of the intact animal to isolated tissue and finally to soluble systems. Regarding the inability of mammalian hormones to act on unicellular organisms, we now have numerous examples were seemingly identical metabolic reactions occurring in vertebrate and invertebrate cells actually differ in fine or gross details, *e.g.*, in nature of the enzyme catalyst, in cofactor requirement, or even in the actual chemical reaction. Moreover, in some cases the amounts of hormone necessary for action can be calculated to be in the range where interaction with a single enzyme or other cell constituent would be possible. In such calculations consideration must be given to the amounts of hormone required for maximal or half maximal effect, possibility of fixation of hormone and the possibility that when steady states exist, very small changes in an enzyme or other cell component may result in drastic overall changes. This point will not be pursued here except to mention Clark's outstanding contributions in this area (1), and to add that if we compare his calculations for the molecules of neurohormones necessary for half maximal activity, to the number of molecules of an enzyme per unit weight of tissue, the figures are of the same order of magnitude. It seems likely that a useful working hypothesis will include the possibility of hormonal action at a (soluble) molecular level.

The second major problem is that of correlating results of *in vitro* experiments with the classically described and reproducible actions of the hormone *in vivo*. In certain cases interesting effects of hormones on broken cell preparations have been reported; however, it often is not apparent what relation these observations have to the known action of the hormone *in vivo*. Therefore, two large gaps exist in the analysis of hormonal action (gaps which experiments to date have not closed) *i.e.*, what specific metabolic events are some hormones influencing in the intact animal and what is the physiological significance of the observations made with broken cell preparations.

Despite the difficulties which have been encountered in analysis of hormonal action, considerable progress has been made toward understanding how these agents may regulate metabolic processes. We have now come to realize that almost all known metabolic reactions are

catalyzed by enzymes, and we know that these enzymatic reactions are influenced not only by the enzymes but by various cofactors which may accelerate the reactions, and by other factors which may either accelerate or depress the rate of the reactions. The biochemical analysis of metabolic reactions has proceeded rapidly in recent years and may now have reached a stage where in selected cases a more exact analysis of hormonal action may be anticipated. For example, recent reports by Villee *et al.* (2) of a stimulatory effect of estradiol on a soluble DPN-linked *iso*citric dehydrogenase are of great interest.

At this time it is intended to discuss some approaches and methods which have contributed to our knowledge of hormonal action with emphasis upon approaches which may lead to understanding of the intimate nature of hormonal action. Certain reports dealing with hormonal effects on enzymes or on broken cell preparations will not be considered because the physiological significance of the results is not clear. Future analysis of these effects and correlation of these findings with *in vivo* findings may be very fruitful, however.

## PREPARATIONS AND APPROACHES FREQUENTLY EMPLOYED IN ANALYSIS OF HORMONE ACTION

### In vivo *preparations*

Much of the knowledge that we possess regarding hormonal action has been obtained from studies of relatively intact animals and in some cases the knowledge is essentially limited to such studies.

The limitations of studies with intact animals have been evident and it seems unlikely that definitive analysis of hormone action will be gained from these studies alone. The difficulties in analysis arise in part because the hormonal control mechanisms are highly integrated, because compensatory mechanisms of various types are brought into action when hormonal imbalance is present or created, and because the complex reactions within a given organ may be influenced by the metabolism or metabolites of other organs. These three difficulties may not allow a decision as to whether a given hormonal effect is a primary effect or a secondary effect.

Nevertheless, it is a logical necessity to have results observed in the intact animal correlated with *in vitro* results in order to establish the physiological significance of *in vitro* findings.

### In vitro *experiments*

*Intact cell preparations.*

*Perfused organs.* — Perfused organ preparations are intermediate in nature between intact animal preparations and other *in vitro* preparations. In some cases it is possible to maintain the organ cells in relatively well preserved condition for some time, and at the same time the organ cells may be separated from the influence of other organs. Perfused organs have been used to study the action of several hormones with varying degrees of success. Although technically some perfusions are not simple, such preparations may be of considerable value in selected cases, and perhaps organ perfusion should be used more widely. If slicing or more drastic treatment of cells results in a loss of response of the cells to the hormone, and if a latent period before hormonal action is anticipated, use of perfused organ may yield valuable information which could not be obtained readily with slices or homogenates.

*Other intact cell preparations.* — a) *Slices.* Tissue slices have been widely used and are valuable preparations for the study of relatively intact cells. A number of slices can be obtained from a single organ, thus increasing the number of experiments which can be carried out with a single animal and consequently the effect of individual animal variation can be minimized. Tissue slice techniques have been reviewed recently (3) and will not be discussed in detail here. The slice technique has been applied especially to liver, brain and kidney, but slices from numerous other organs have been studied.

b) *Others.* Preparations containing relatively intact muscle cells have posed special difficulties. Cells present in muscle slices are rather extensively damaged especially when skeletal muscle has been used. The rat diaphragm has been used widely and some factors in the use of diaphragms have been reviewed by Krahl (4) and by others. Fiber bundle preparations of skeletal muscle have been obtained (5) and small single muscle preparations have been used, *e.g.*, a single frog muscle. Occasionally muscle minces have been used (6).

*Broken cell preparations.*

*In vitro* experiments utilizing intact cells are simpler in many respects than experiments involving intact animals. Nevertheless, the activities of the intact cell are numerous and complex; therefore, the natural tendency has been to break the cells and search for hormonal effects in broken cell preparations. Permeability problems are less obvious in these preparations, although the desirability of removing cell membrane permeability relations remains to be proved. Even though cell membranes may be broken, many preparations contain particles where membrane phenomena are operative and in these cases it is apparent that complex relations exist even though some simplification of preparation has been achieved. Preparation of homogenates, particulate fractions from homogenates, and extraction of enzymes from tissue have been reviewed recently (7-9). The potential value of studies with broken cell preparations is obvious; precise analysis of hormonal action in such systems should be simpler and more conclusive. Extrapolation of hormone effects in these preparation to the physiological action of the hormone in the intact animal must be done with extreme care, however. A very simple example of the difficulty of such extrapolation was encountered when insulin was added to a reaction mixture containing purified phosphoglucomutase (10). Zinc free insulin stimulated the activity of this enzyme, but it soon became apparent that the enzyme was very sensitive to traces of heavy metals and that zinc free insulin was a moderately good metal binding agent and, in fact, was somewhat more effective than crystalline serum albumin.

## EXAMPLES OF ANALYSIS OF HORMONAL ACTION

### *The action of insulin*

The action of insulin has been studied so intensively that it will be discussed briefly here in order to illustrate the difficulties involved in analysis of hormone action and to indicate the progress that has been made. No attempt will be made to evaluate in detail current investigative work.

In the animal, lack of insulin results in a disturbed metabolism of carbohydrate, fat and protein; in some cases changes in body fluid and salts are evident. In man the daily administration of 2 mg. of insulin frequently corrects these disorders of metabolism. This amount of insulin is indeed small : if this amount of insulin were distributed in 35 kg. of body fluid or tissue, the concentration would be about $1 \times 10^{-8}$ M. Obviously, other factors enter into the concentration of insulin in any given area, but the point made now is that very small amounts of this protein can bring about many changes in the intact animal. Search for insulin substitutes and experimental modification of the insulin molecule indicate that the action of insulin is dependent on a specific structure. The action of insulin to date has not been simulated by any compound; only insulin or very slightly modified insulin will correct the classical disorders brought about by insulin lack.

It seems unlikely that insulin, by diverse action, can participate in all the responses noted in intact animals. We know now that some of the disorders noted in the intact animal are secondary events. Exactly which effect(s) of insulin is primary still is debated, but tremendous progress has been made in understanding the complex changes resulting from insulin lack.

In this discussion it will be impossible to give due credit to the numerous investigators who have gathered this information. A good share of the information has resulted from the work of individuals who were studying physiological or biochemical mechanisms and not insulin action *per se*. These studies have led to our current ideas of physiological-biochemical mechanisms involved in the metabolism of cells; in many cases basic enzymatic reactions have been clarified. Of special importance to the problem of insulin action is the knowledge that the metabolic reactions are interrelated. The metabolism of carbohydrate, proteins and fats proceeds through common steps and utilization by cells of one type of substrate may influence the utilization of another type of substrate. The metabolic reactions are so interrelated that an effect of insulin at a specific site could explain the complex changes related to insulin action in the intact animal.

Investigators studying the action of insulin *per se* have utilized all of the preparations outlined in a previous section and, in fact, have used some preparations not mentioned, *e.g.*, embryonic chicks. Study of these preparations has yielded much valuable information, although results with broken cell preparations have been rather disappointing. Results with intact animals, eviscerated animals, isolated rat diaphragms, etc., can now be correlated in a fairly satisfactory theory (11). Most, if not all, of the diverse effects noted in the intact animal are compatible with the idea that the primary effect of insulin is to promote the utilization of glucose. Time does not permit development of this theory but a few landmarks will be noted. (For reviews of insulin action, see 12-16). As early as 1912, Knowlton and Starling (17) noted that heart-lung preparations from diabetic dogs used less glucose than similar preparations from normal dogs. Banting and Best (18) noted that the insulin which they extracted promoted the removal of glucose from the blood. Mann *et al.* (19) and others (20) observed that insulin increased the rate of glucose disappearance in a hepatectomized animal. Gemmil (21) employed the isolated rat diaphragm preparation and demonstrated that addition of insulin to such preparations resulted in an increased utilization of glucose and deposition of glycogen. The response of the rat diaphragm to insulin has been studied extensively in recent years (4, 22). de Duve *et al.* (23) demonstrated that hexosemonophosphate content of muscle was increased by insulin action under conditions where blood sugar levels were maintained at normal levels. In addition to these developments which have shown that insulin promotes the utilization of glucose, much evidence has accumulated that disorders in the metabolism of fats and protein caused by insulin insufficiency may be secondary to the disorder in glucose utilization. In brief summary, analysis of insulin action on intacts cells *in vivo* and *in vitro* has been extensive and apparently fruitful.

It appears that the general difficulty in analysis of insulin action exists in the establishment of reproducible hormonal effects in broken cell preparations. Cell activities are so complex that to date investigators find it difficult to distinguish a specific action on glucose transport across cell membranes from an action on a hexokinase enzyme. Indeed it has been difficult to distinguish whether an action on glucose transport (or on hexokinase) is a primary action or possibly secondary to another effect on cell activity which may be rather specifically linked to glucose transport and utilization. The chief aim of this brief section on insulin action is to indicate the difficulties which arise when analysis of hormonal action is handicapped by an erratic or negative response of broken cell preparations to the hormone.

### *Action of epinephrine and glucagon on glycogenolysis*

The numerous and apparently diverse effects of epinephrine in the intact animal have been described in recent pharmacological texts (24), and recent reviews of glucagon action are available (16, 25, 26, 27). Glucagon was studied and named by Kimball and Murlin in 1923 (28). In this discussion only occasional reference will be made to the action of these agents in intacts animals. It should be clear at the outset, however, that epinephrine stimulates glycogenolysis in liver and muscle of intact animals, while the primary established action of glucagon in the intact animal consists of a stimulation of glycogenolysis in liver.

#### *Acceleration of glycogenolysis in liver slices.*

*Establishment of suitable experimental preparation.* — Glucagon and epinephrine increased glycogen breakdown and glucose output when added to liver slices *in vitro*.

It was possible to obtain graded responses to increasing concentrations of these agents until a maximal response was obtained. Liver slices therefore were suitable for assay purposes and amounts of agents necessary for half maximal stimulation were determined (29). Table I summarizes the relative activities of several sympathomimetic amines when the activities were determined by the liver slice method. Results of these determinations are compared to the relative hyperglycemic activities determined in the intact animal by McChesney *et al.* (30) and it can be seen that the activities in the liver slice test system parallel the order of hyperglycemic activity in the intact animal. Of these compounds *l*-epinephrine was most active in the liver slice test system with half maximal stimulation occurring at about 1 part per 10 million in the standard test system. Maximal stimulation of glycogenolysis occurred when the concentration of *l*-epinephrine was about 1 part in 4 million or higher, at which concentrations the rate of glycogen breakdown was usually increased two-fold over controls. These and other experiments indicated that liver slices were suitable preparations for study of the action of glucagon and epinephrine on glycogenolysis.

*General site of action in slice preparations.* — a) *Overflow* vs. *extrusion*. Necessity of cell structure for demonstration of significant hormonal effects has suggested that cell membrane activities should be considered in analysis of hormone action. The increased glucose output from slices could have resulted from an increased production of glucose within the cell accompanied by increased glucose concentration in the cells with subsequent increased flow of glucose to the medium surrounding the slices. It was also possible that the increased glucose output could have resulted from an active extrusion of glucose from the cell. Such a process would lower the glucose in the interior of the cell and this could result in a shift of the overall reactions toward the production of more glucose. However, it could be shown that the increased glucose output from the slices was the result of increased production of glucose within the cell followed by overflow to the medium simply by measuring the glucose content of the cells during the period of hormonal activity. The cells stimulated by glucagon or epinephrine contained much more glucose than the controls (31). This analysis did not lead to the conclusion that the primary action of the hormones had been separated from cell membrane phenomena, but did show that the increased glucose release from slices was not due to an extrusion process.

TABLE I

*Relative activities of sympathomimetic amines* in vitro *and* in vivo

| Sympathomimetic amine | Relative activity | |
|---|---|---|
| | Liver slice assay | Intact animal assay |
| *l*-epinephrine | 100 | 100 (*) |
| *l*-norepinephrine (levarterenol) | 16 | 12 (*) |
| *d*-epinephrine | 16 | |
| *d*-norepinephrine | 2.0 | 0.6 (*) |
| Amphetamine | 0.0 | 0.0 |

(*) Determined by McChesney *et al.* (30).

b) *Phosphorolysis* vs. *hydrolysis*. The increased breakdown of glycogen resulting from the action of these hormones could have resulted either from a stimulation of hydrolysis of glycogen or from a stimulation of phosphorolysis of glycogen. It was possible to establish that phosphorolysis was stimulated rather than hydrolysis. Several results led to this conclusion; most important of these was the finding that the increased breakdown of glycogen noted on addition of epinephrine or glucagon resulted in the formation of monosaccharide and not in the formation of larger carbohydrate units which would be expected if analyse activity had been stimulated (30).

c) *Stimulation of phosphorylase system*. Three enzyme systems participate in the conversion of glycogen to glucose *via* phosphorylated intermediates, *i.e.*, phosphorylase, mutase and phosphatase systems. They are termed enzyme systems because various factors besides the enzyme protein may alter the reaction, *i.e.*, coenzymes, inhibitors, substrate levels, etc., may play important roles in these reactions. A few experiments will be summarized here which were designed to determine which of the three systems was stimulated by glucagon and epinephrine.

When liver slices were incubated with media containing 0.02 M phosphate pH 7.0 to 7.5, breakdown of glycogen to glucose was a predominant reaction and net synthesis of glycogen from glucose could not be demonstrated. It was shown that the mutase system and the phosphatase system could dispose of much more substrate than that furnished by the phosphorylase system and it appeared that the phosphorylase system limited the rate of glycogenolysis in slices. In homogenates phosphorylase definitely was rate limiting in the conversion of glycogen to glucose.

These results indicated that the effect of glucagon and epinephrine was on the phosphorylase system but did not prove that this was the site of action. Even though the mutase and phosphatase systems in the slice were not saturated by substrate, if stimulated, they could possibly speed up the rate of glycogen breakdown by removing glucose-1-phosphate or glucose-6-phosphate at a faster rate. This would result in a lower level of glucose-1-phosphate or glucose-6-phosphate in the cells stimulated by glucagon or epinephrine. On the other hand, if the phosphorylase system were stimulated, the concentration of glucose-1-phosphate should be increased. If the concentration of glucose-1-phosphate increased, we would expect glucose-6-phosphate to increase to some extent, since glucose-1-phosphate is rapidly converted to glucose-6-phosphate; certainly, it should not decrease if the action of glucagon and epinephrine were on the phosphorylase system. In order to settle this question it was necessary to measure the concentrations of hexose phosphates in the cells.

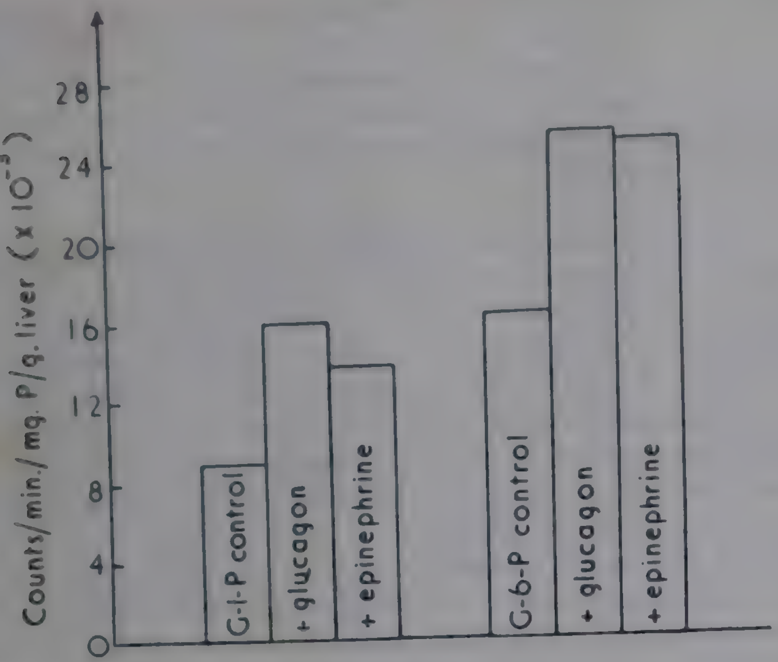



Fig. 1. — Analysis of glucose-1-phosphate and glucose-6-phosphate in liver slices. Isotopic hexose phosphates were formed by incubation of liver slices with radioactive inorganic phosphate. After addition of non-radioactive carrier, the intermediates were purified and radioactivity was determined. From Sutherland (31).

Liver slices without and with addition of glucagon or epinephrine were incubated with buffer containing isotopic inorganic phosphate, and experimental conditions were arranged so that the tissues could be fixed rapidly at a suitable time. At this time certain amounts of the labeled intermediates (glucose-1-phosphate and glucose-6-phosphate) were present in the fixed slices. This amount was measured after mixing with a known large amount of either glucose-1-phosphate or glucose-6-phosphate, followed by careful isolation of the intermediate and determination of the isotope content. Figure 1 shows the results of such an experiment where liver slices were incubated with radioactive inorganic phosphate. After 25 minutes of incubation without and with glucagon or epinephrine, the incubation was stopped by placing the tubes in a boiling water bath at which time a large amount of either glucose-1-phosphate or glucose-6-phosphate (non-radioactive) was added to the tubes. In those where glucose-1-phosphate was added, the isotopic inorganic phosphate was removed by addition of magnesia mixture, then after each of several crystallizations of dipotassium glucose-1-phosphate, non-radioactive inorganic phosphate was added and then was removed by magnesia mixture. In those tubes where glucose-6-phosphate was added, the procedure was similar except that the inorganic phosphate was removed as the barium salt and the barium salt of glucose-6-phosphate was obtained after the addition of alcohol. It can be seen that the amount of glucose-1-phosphate, as indicated by the radioactivity, was distinctly higher in those slices which were incubated with glucagon or epinephrine. This finding was confirmed in other experiments where more rigorous isolation procedures were used. The glucose-6-phosphate was also higher in those slices incubated with glucagon or epinephrine, and this finding was confirmed by direct chemical analyses of slices. These findings led to the conclusion that glucagon and epinephrine stimulate the phosphorylase system in liver slices (31).

*Relation of active liver phosphorylase in inactive liver phosphorylase.*

*Effect of glucagon and epinephrine on enzyme concentration.* — Further investigation of the hormonal effect on the phosphorylase system has shown that the concentration of active enzyme in liver slices was increased by glucagon and epinephrine. This was demonstrated directly by measurements of phosphorylase activity in homogenates or extracts prepared from slices which were pre-incubated without or with these agents. Figure 2 summarizes two experiments which illustrate this point (32). The time of pre-incubation is written on the bars : C refers to control slices ; E to slices incubated with epinephrine ; and F to slices incubated with glucagon. The slices at zero time contained all of the phosphorylase in its active form. This was usually the case when no special precautions were taken in the killing of the animal and the removal of the liver. The first experiment shows the loss of phosphorylase activity when slices were incubated for 30 minutes at 37° C. The homogenates from slices incubated with epinephrine or glucagon had considerably more phosphorylase activity than the control. The second experiment shows that epinephrine can restore phosphorylase activity very rapidly once it has decreased. After 20 minutes of incubation at 37° C., the phosphorylase activity of the control had fallen considerably, and there was only a slight additional decrease of phosphorylase during the next 20 minutes. After 20 minutes of incubation, epinephrine was added to some of the slices. Within

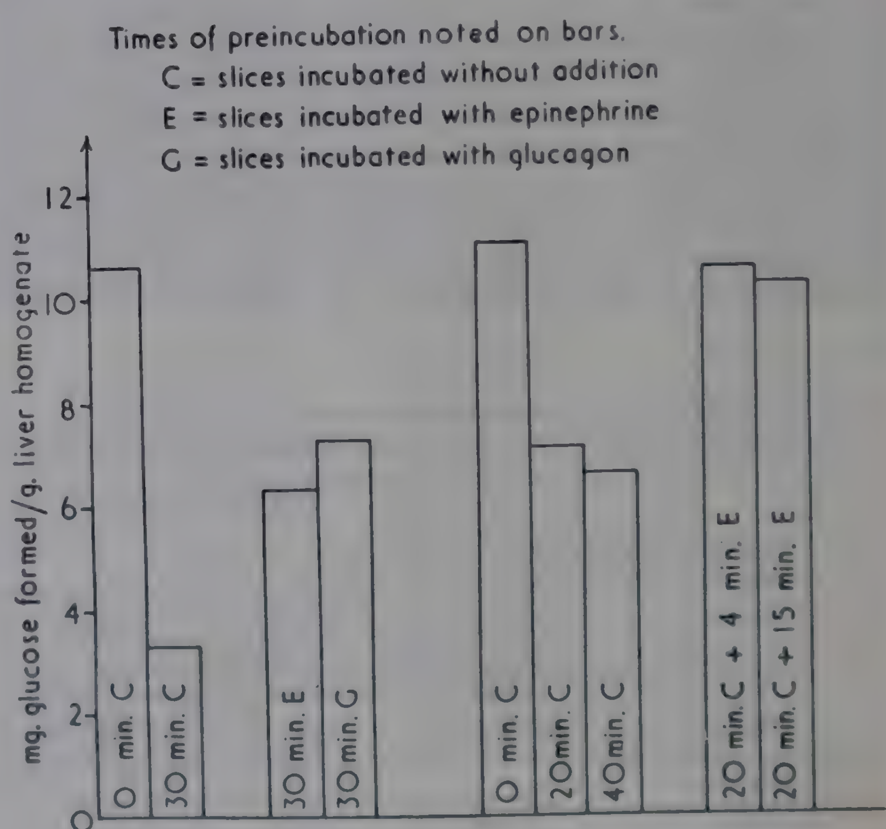



Fig. 2. — Phosphorylase activity in homogenates prepared from preincubated rabbit liver slices. The rate of glucose formation was measured at pH 7.4 in 0.04 M phosphate buffer and 1.0 % glycogen. From Sutherland (32).

4 minutes, the phosphorylase activity had almost returned to the original value, and the effect was as large after 4 minutes of incubation as after 15 minutes of incubation with epinephrine. The same results were obtained when glucagon was used in place of epinephrine. It should be pointed out that this increase in the phosphorylase content of liver slices could also be demonstrated when phosphorylase was tested in the direction of synthesis, *i.e.*, formation of polysaccharide from glucose-1-phosphate in homogenates prepared from slices.

These and related experiments demonstrated that epinephrine and glucagon stimulated glycogenolysis in liver slices by promoting an increase of active phosphorylase. The level of phosphorylase in liver slices represented a balance between enzymatic inactivation and reactivation to the active form. The change from the inactive form to the active form was very rapid and the amount of active enzyme in slices was doubled within a few minutes following addition of glucagon or epinephrine. At this stage the reactivation of the inactive enzyme to the active form could be demonstrated only in intact tissue, although once reactivation within the slice had occurred the increased amounts of active enzyme could be demonstrated in homogenates, extracts or in fractions of extracts obtained by ammonium sulfate precipitation.

Since reactivation was not obtained in homogenates, the precise role of the hormones in the activation process could not be readily defined at this time. In order to study the nature of the changes in the phosphorylase molecule it seemed advisable to purify liver phosphorylase and the enzyme which inactivated it, since the inactivation reaction did occur in homogenates and could be studied in cell extracts.

*Enzymatic inactivation of liver phosphorylase.* — Liver phosphorylase in homogenates or in purified fractions was rapidly inactivated by an enzyme temporarily designated liver phosphorylase inactivating enzyme. The product of the reaction was termed inactive phosphorylase rather than the *b* form, since enzymatic activity was not restored when tested in the presence of adenosine-5-phosphate. Liver phosphorylase and the enzyme inactivating it (33) were prepared in purified form from dog liver (45, 46). Purified liver phosphorylase was inactivated enzymatically and a search was made for small fragments that might be released during the reaction. Inorganic phosphate was found to be a product of the reaction and was determined chemically by the method of Fiske and SubbaRow or by the Lowry-Lopez method (34). The enzymatic liberation of phosphate was determined not only by chemical measurements but by radioactivity when radioactive liver phosphorylase was used as a substrate, prepared as described in a later section. Formation of inorganic phosphate paralleled the formation of inactive liver phosphorylase as shown in figure 3. The phosphate present in the molecule of liver phosphorylase was very firmly attached to the protein moiety and remained with the protein on precipitation with trichloroacetic acid; after enzymatic inactivation the phosphate appeared in trichloroacetic acid supernatants. In this experiment 1 mole of inorganic phosphate was formed per 120 000 grams of protein, or

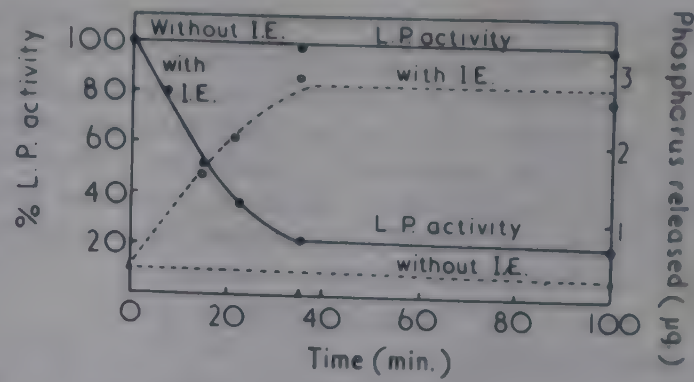



Fig. 3. — Appearance of inorganic phosphate in the supernatant fluid of liver phosphorylase precipitated with trichloroacetic acid coincident with enzymatic inactivation. From Sutherland and Wosilait (34).

approximately 2 moles of phosphate per mole of enzyme. No production of pentose compounds, phenolic type compounds, peptides or ultraviolet absorbing materials was detected. Furthermore, no proteolytic activity was observed when the inactivating enzyme was incubated with several proteins. The inactive form of the enzyme sedimented in the analytical ultracentrifuge at the same rate as the active form; in either case only a single peak was noted.

Table II

*Possible substrates incubated with inactivating enzyme*

| P released | No P released |
|---|---|
| Liver phosphorylase | *Proteins* |
| | Casein |
| α-Casein | α-Casein |
| | Ovalbumin |
| Phosvitin | |
| | *Non-proteins* |
| | α-Glycerophosphate |
| | *p*-Nitrophenyl phosphate |
| | 5-AMP |
| | ←ADP |
| | ←ATP |
| | Creatine phosphate |
| | Phosphoenolpyruvate |
| | Glucose-1-phosphate |
| | Fructose-1, 6-diphosphate |
| | Ribose-5-phosphate |

No phosphatase activity was noted when inactivating enzyme was incubated with a number of simple organic phosphorus-containing compounds or with some phosphoproteins (table II), but small amounts of phosphate were liberated from certain other phosphoproteins, namely phosvitin and α-casein. The activity of the enzyme on phosvitin and on liver phosphorylase paralleled one another when the enzyme preparation was subjected to various treatment. Both activities were inhibited by 0.01 M NaF and salts, for example, 0.15 M NaCl, and neither activity was stimulated by magnesiums ions. The activities were parallel following heat treatment, *i.e.*, at pH 4 for 15 minutes at 37° C. all

the activity was lost, while at pH 8 for 15 minutes at 50° C. most of the activity was retained. Only a small per cent of the total phosphate of phosvitin was released on long incubation so it was not clear that phosphoserine proteins were the necessary substrates. The organic phosphate portion of liver phosphorylase is currently under investigation. The inactivation reaction at present may be written in the following form :

$$\text{Liver phosphorylase} \xrightarrow{\text{LP-phosphatase}} \text{Liver dephospho-phosphorylase} + \text{inorganic phosphate.}$$

*Conversion of dephosphophosphorylase to phosphorylase in liver slices.* — a) *Glucagon and epinephrine on incorporation of phosphate into phosphorylase.* Since inorganic phosphate was released during enzymatic inactivation of phosphorylase, it seemed probable that the conversion of dephosphophosphorylase to phosphorylase consisted of a phosphorylation of the inactive form. Liver slices were known to incorporate inorganic phosphate rapidly into various esters (which might be capable of donating phosphate to the acceptor group of the inactive form) and on this basis experiments were designed to see if slices were able to incorporate phosphate into the inactive enzyme during the process of activation.

The following experiment summarized in figure 4 demonstrated that $^{32}$P-orthophosphate was incorporated rapidly into phosphorylase and that the amount of labeled phosphate present in the slice phosphorylase was much greater when slices were incubated with glucagon or epinephrine. Dog liver slices were preincubated for 15 minutes with buffer containing $^{32}$P-orthophosphate to permit inactivation of phosphorylase and also entry of the labeled phosphate, then were further incubated for 5 minutes without and with the addition of epinephrine (E) or glucagon (G). At the end of the incubation the medium was decanted and the slices were homogenized in the presence of carrier liver phosphorylase. The bars at the left indicate the level of phosphorylase activity in the slices before carrier addition and the bars at the right indicate radioactivity of the purified phosphorylase prepared from the homogenates. Purified samples from homogenates E and G contained 5 to 6 times as many counts as the control sample. The cross-hatched portion of the bars represents the portion of the radioactivity that became soluble in trichloracetic acid after enzymatic inactivation of the samples. The phosphate present in liver phosphorylase did not exchange with substrate phosphate as the enzyme acted catalytically; this was shown by a series of experiments which will not be detailed here.

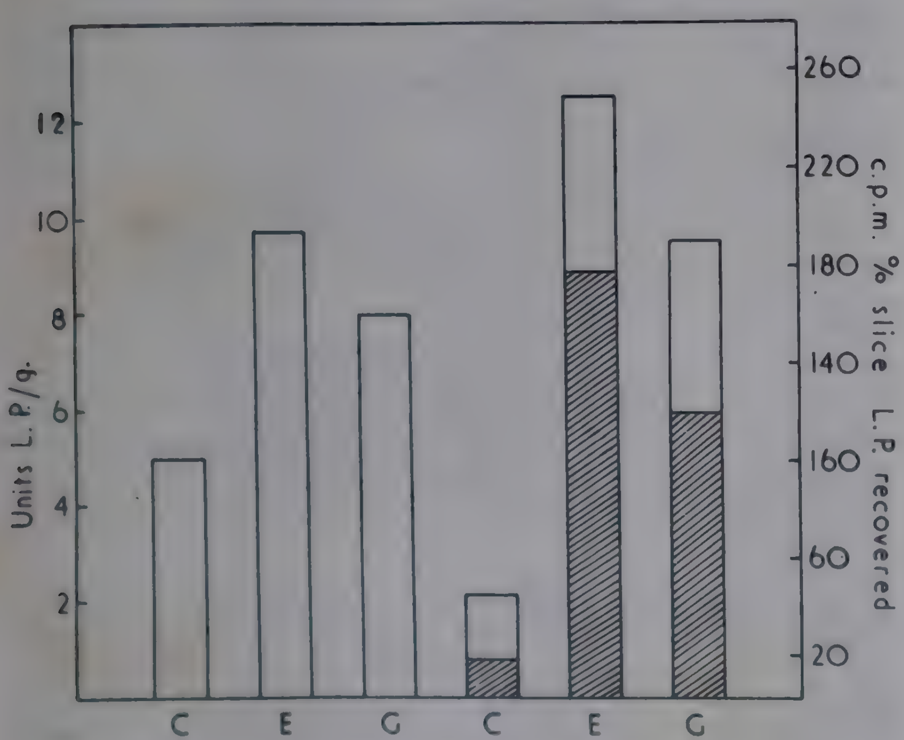



Fig. 4. — Effect of epinephrine and glucagon on $^{32}$P incorporation into dog liver slice phosphorylase. Three portions of slices were incubated for 20 minutes at 37° C. in a medium containing 0.15 M glycylglycine + 0.001 M phosphate buffer (pH 7.4) and 1 mC of $^{32}$P. Epinephrine or glucagon was added to two portions (E and G) five minutes before end of incubation. The cross-hatched portion of bars at right refers to radioactivity made trichloroacetic acid-soluble after enzymatic inactivation of final preparations. L. P. = liver phosphorylase.

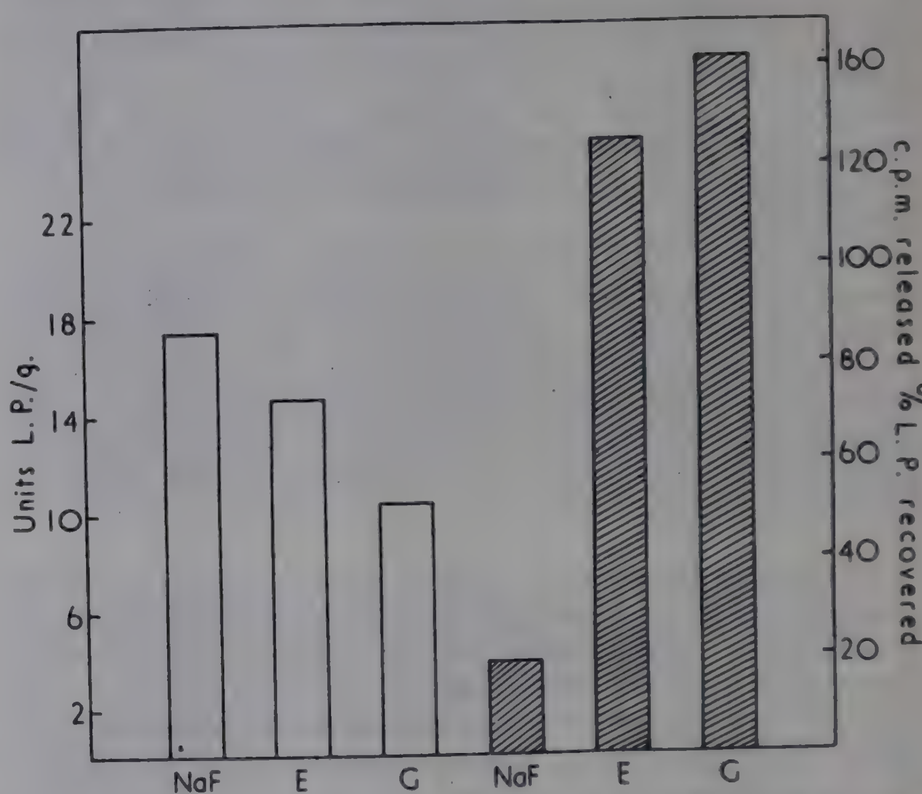



Fig. 5. — Comparison of the effects of NaF, epinephrine and glucagon on the incorporation of $^{32}$P into liver slice phosphorylase. Three portions of slices were incubated 15 minutes in a medium containing 0.15 M NaCl + 0.02 M glycylglycine + 0.001 M phosphate buffer (pH 7.4) and epinephrine (E) or glucagon (G), or containing 0.15 M NaF in place of NaCl. Five minutes before end of incubation, 1 mC of $^{32}$P was added. Cross-hatched bars at right refer to radioactivity made trichloroacetic acid-soluble after enzymatic inactivation of final preparations. L. P. = liver phosphorylase.

*Comparison of glucagon and epinephrine with an inhibitor of inactivating enzyme.* — With the additional knowledge and techniques now available, it was possible to study of glucagon and epinephrine exerted their action by stimulation of the kinase system responsible for the donation of phosphate to dephosphophosphorylase in slices.

The incorporation of radioactive phosphate into phosphorylase during incubation with epinephrine or glucagon was compared to incorporation in the presence of fluoride, a known inhibitor of the inactivating enzyme whose

addition to slices also results in an increased level of phosphorylase. Results of one such experiment are summarized in figure 5. The bars to the left represent phosphorylase activity of the slices at the end of the experiment; the bars to the right represent the radioactivity of phosphorylase purified from the slices as determined by release of phosphate on enzymatic inactivations. Fluoride maintained phosphorylase at a high level during incubation and during this time very little radioactive phosphate entered the phosphorylase protein. Epinephrine and glucagon likewise maintained the level moderately well, but ih these cases the radioactive phosphate was incorporated readily into the phosphorylase protein. This indicated that fluoride inhibited the inactivating enzyme thereby preventing the removal of phosphate from the existing phosphorylase; consequently the added radioactive phosphate could not be incorporated in phosphorylase. Epinephrine and glucagon on the other hand acted by a different mechanism which indicated the effects resulted from a stimulation of the phosphate donor system.

It was concluded that dephosphophosphorylase had been formed during incubation with NaCl and on addition of fluoride the reformation of active enzyme was evident, since the inactivating system was now inhibited and limiting thus permitting the kinase action to become predominant. As this net formation of active enzyme proceeded in the presence of fluoride, radioactive phosphate was incorporated. Thus it appeared that the presence of dephosphophosphorylase was required for the incorporation of radioactive phosphate into phosphorylase. The fourth pair of bars indicate that when the inactivating enzyme was inhibited by fluoride, the incorporation of phosphate was inhibited even in the presence of epinephrine, since only small amounts of dephosphophosphorylase had been formed.

*Conversion of dephosphophosphorylase to phosphorylase with soluble phosphokinase preparations.* — It was deduced from the experiments summarized above that a kinase was involved in the conversion of dephosphophosphorylase to phosphorylase. The protein-phosphate

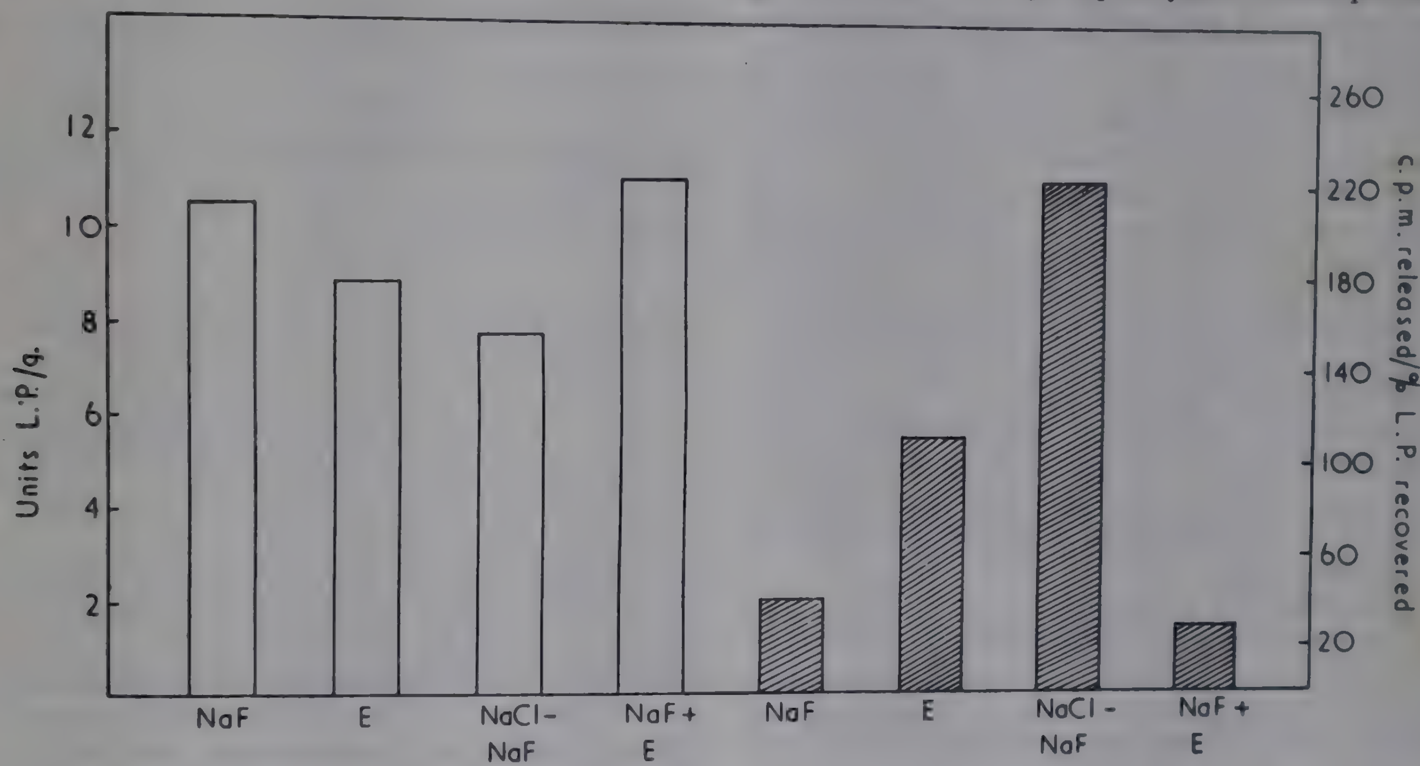



**Fig. 6.** — Effect of adding $^{32}P$ and NaF simultaneously on incorporation of $^{32}P$ into liver slice phosphorylase. Four portions of slices were incubated 17 1/2 minutes under the conditions of figure 5. One mC of $^{32}P$ was added to each portion 7 1/2 minutes before the end of incubation; at the same time the NaCl medium of one portion was decanted and replaced with NaF-containing medium. Cross-hatched bars at right refer to radioactivity made trichloroacetic acid-soluble after enzymatic inactivation of final preparations. L. P. = liver phosphorylase.

Subsequent experiments supported this interpretation (35), as shown by an example summarized in figure 6. In this case the same experimental procedure was employed with only slight variation. In one flask (3rd bar) the slices were incubated with NaCl for 10 minutes, then both NaF and radioactive phosphate were added simultaneously. At this time the concentration of active phosphorylase in the slices had fallen to a low level. Addition of fluoride for the final 7-½ minutes increased the concentration of active phosphorylase very substantially, and during this period radioactive phosphate was readily incorporated into phosphorylase protein as can be seen from the third cross-hatched bar.

bond was firm and was not broken by precipitation with trichloroacetic acid nor by short incubation with hot dilute HCl or hot dilute NaOH and the phosphate did not exchange when incubated for prolonged periods with inorganic phosphate.

Studies on the reactivation process in broken cell preparations led to the finding of a phosphokinase (*)

(*) This enzyme has been named liver dephosphophosphorylase kinase, thus conforming to current terminology. For convenience phosphokinase is used as an abbreviation. It is pointed out that the use of the term kinase also conforms to an early definition where a kinase was a substance which transformed a zymogen to an enzyme.

capable of transferring phosphate to dephosphophosphorylase. This enzyme was purified more than 50-fold following the procedure summarized in table IV. Early fractionation steps separated the three enzymes rather well and permitted the preparation of the three enzymes from one homogenate. Dephosphophosphorylase kinase required adenosinetriphosphate and magnesium ions for the conversion of its substrate to the active enzyme. Requirement for ATP and $Mg^{++}$ became evident simply by diluting the homogenate into a range suitable for assay (47).

The assay system which was adopted contained (as final concentrations) $1 \times 10^{-3}$ M ATP, $2.5 \times 10^{-3}$ M $Mg^{++}$, $2.0 \times 10^{-2}$ M NaF, buffer, and purified liver dephosphophosphorylase. From results in this assay system, it was possible to calculate that there was enough phosphokinase in liver to activate all the phosphorylase in liver in a very short time — probably in one minute. This degree of activity is sufficient to implicate this enzyme in the rapid response of slice phosphorylase concentration to glucagon and epinephrine.

During the progress of this work, Fischer and Krebs (34) reported the presence of an enzyme from rabbit muscle which catalyzed the conversion of rabbit muscle phosphorylase *b* to phosphorylase *a* in the presence of ATP and manganous ions. A recent report from the Cori laboratory has described the PR enzyme action as a phosphorylase rupturing action (37). It seems possible that the interconversions of phosphorylase in liver and muscle have some similar features, although the several enzymes involved differ physically or in other respects. The three enzymes discussed above have been purified from dog heart muscle to varying degrees in this laboratory. Highly purified inactivating enzyme from heart appears similar to the one from liver, and the very active phosphokinase from heart readily converts liver dephosphophosphorylase to the active form in the presence of ATP and magnesium ions.

Effects of glucagon and epinephrine on soluble phosphokinase have been obtained under certain conditions but have been variable. The work is too recent to present a clear picture, especially since co-factor requirements are present and inhibitory factors or processes also are operative in broken cell preparations.

In summary of this section, active liver phosphorylase is converted to an inactive dephosphophosphorylase by the inactivating enzyme which appears to be a rather unusual phosphatase.

Dephosphophosphorylase is converted to the active enzyme by an enzyme system in which dephosphophosphorylase kinase, $Mg^{++}$, ATP and other factors are involved. In slices glucagon and epinephrine increase the amount of active enzyme possibly through this system. For more exact localization additional investigation will be required.

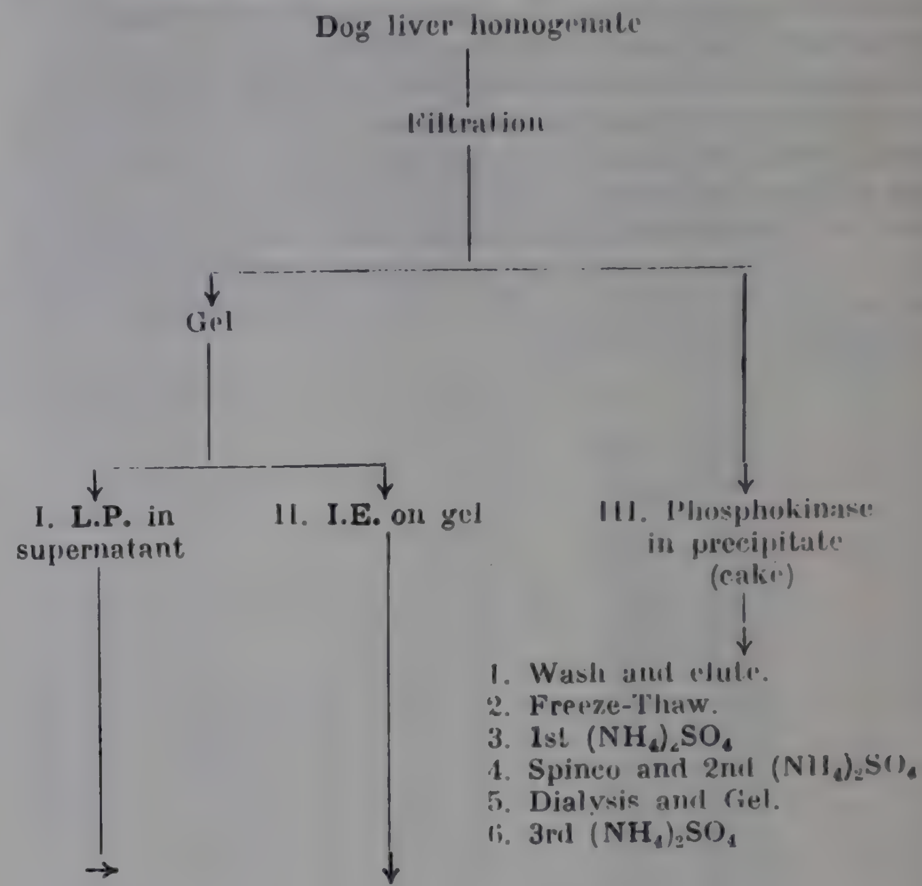
## DISCUSSION

Analysis of the action of epinephrine and glucagon has progressed to the stage where it can be stated that their action results in the activation of an enzyme (liver phosphorylase). The increased amounts of active enzyme which results from their action can be demonstrated in purified soluble protein fractions. It seemed likely that some portion of the phosphokinase system in liver slices was stimulated by these agents.

However, if we reconsider the two general difficulties in analysis of hormone action that were posed in the introduction, we realize that the analysis is incomplete. First of all, it should be clear that the action of glucagon

TABLE IV

*Scheme for purification of liver phosphokinase*




and epinephrine on a soluble clearly defined system has not been soundly established. Moreover, the overall action of glucagon and epinephrine so far has appeared identical. It is well known that epinephrine action on muscle can be readily demonstrated while this is not the case with glucagon. Ellis *et al.* (38) have reported that adrenergic blocking agents are able to block the hyperglycemic response to epinephrine without blocking the hyperglycemic response to glucagon. Cornblath (39) has reported a difference in response of liver slices to epinephrine and glucagon when tested in the presence of ephedrine. It seems likely that further analysis will reveal differences in the mechanism of action of these two agents.

Secondly, the *in vitro* effects have not been well correlated with or extrapolated to the *in vivo* effects. de Duve (25) has pointed out the apparent irreversibility of the epinephrine and glucagon effect in liver slices, even though the enzyme which is activated catalyzes a reversible reaction. Riesser (40) and others (41, 42) have reported that epinephrine causes a breakdown of diaphragm glycogen under conditions where glucogen would otherwise be synthesized. Certain reports of glycagon or epinephrine action on fatty acid metabolism in liver slices have not been correlated with the effect on phosphorylase (43). No attempt has been made as yet to relate the effect of

epinephrine on glycogenolysis to its effect on muscle. It is possible that the phosphokinase system which catalyzes the phosphorylation of inactive liver phosphorylase can catalyze the phosphorylation of one or more different proteins. In any event it is obvious that analysis of the action of glucagon and epinephrine is complete.

In conclusion, it seems possible that the approaches used in the analysis of action of glucagon and epinephrine can be applied to the study of other hormones. For example, Haynes *et al.* (44) have reported that ACTH stimulated steroid output from adrenal slices. From this basic observation Haynes has proposed a plan of analysis similar to that discussed here. It would appear desirable in some cases to pursue analysis of hormone action with intact cells *in vitro* since in any case such studies will be helpful in evaluation of effects noted in broken cell preparations.

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# Certain difficulties in the analysis of hormone action

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(*Received* 19 *September* 1955)

Dr. Sutherland has lucidly discussed a number of the difficulties inherent in the study of the mechanism of hormone actions upon the processes of intermediary metabolism, and has indicated some of the means he has employed to overcome them. One difficulty not mentioned by him, yet which is almost insuperable, arises from the uncertainty that the isolated and, in some cases, highly purified endocrine product is indeed the normal active principle at the target organ.

This uncertainty stems from two sources. The first is the obvious possibility that the agent is chemically altered by the death of the tissue from which it is extracted or by the procedures of extraction and purification. Equally difficult to evaluate is the possibility that the hormone contained in the endocrine gland is in some fashion different from the material which this same gland normally discharges into the blood stream. As Dr. Sutherland has mentioned, most of the hormones circulate at very low levels of concentration and this has rendered difficult the resolution of the question : What is the chemical nature of the circulating hormone ? The fact that with certain hormones, such as growth hormone, a time lapse ensues between administration and observed effect suggests the possibility that the substance injected must undergo some biological transformation prior to its effect on the target organ or tissue.

In the case of the pancreatic hormones, insulin and glucagon, an additional complication exists. These hormones, as normally generated, are doubtless first delivered to the liver *via* the portal blood channel. In the case of glucagon, this arrangement is effective in view of the fact that the action of this material is largely if not entirely upon liver glycogen. Bornstein has recently found that a single passage of glucagon solution through a perfused liver effectively removes all the hormone activity from the perfusing fluid.

In the case of insulin, this obligatory trans-hepatic route is more difficult to evaluate. Recent evidence from Hastings' laboratory has given renewed vigor to the belief that insulin as such has no demonstrable direct action upon the metabolism of liver cells. The direct action of insulin is largely confined to its effects upon peripheral tissues, chiefly muscle. Is it possible, or likely, that insulin passes from the portal to the hepatic vein without chemical alteration ? Is the hormone, as delivered to the peripheral tissues, identical with the material discharged by the β-cells of the islets of Langerhans and is this in turn identical with the material secured by acid-alcohol extraction from the pancreas ? The answers to these questions are not known.

What is known, however, is that both experimentally and therapeutically insulin has generally been administered to intact animals into the peripheral circulation, not the portal circulation. An evaluation of possible differences in the responses of muscle metabolism to insulin injected into the portal and peripheral veins might give an indication of the hepatic contribution to insulin action.

Dr. Sutherland has discussed the fact that hormones in general are physiologically active in the intact animal while at remarkably low molar concentrations in the extracellular fluid. This fact at once suggests that these compounds act catalytically. For various hormones it has been proposed that they activate, inactivate or catalyze the synthesis of enzymes. In the case of insulin, it has been proposed that the hormone in some as yet unknown fashion permits certain sugars access to the intracellular compartment from which they are otherwise excluded. In the special case of thyroid hormone, an action of uncoupling of a physiologically coupled mechanism has been postulated. The low concentrations of physiologically active hormones in the circulating blood might further suggest that the cells of the target organ possess a mechanism of selective concentration of hormone. Some evidence indicating the nature of such a mechanism is at hand from the work of Stadie and his collaborators who have shown that not only muscle but also adipose and mammary tissues possess the capacity of adsorbing and firmly binding insulin from solution. The situation is not dissimilar to that described by the Michaelis-Menten concept that the rate of an enzyme-catalyzed reaction is determined by the instantaneous concentration of an enzyme-substrate complex. Translating this concept into endocrinological terms, it may be postulated that the effect of a hormone is determined by the instantaneous quantity of hormone bound by the effector or target organ.

It is of interest to speculate from the assumption that the response in the target organ is in effect determined by the abundance of target organ acceptor site occupied by hormone, [HT]. Employing a derivation analogous to that used by Michaelis and Menten to describe enzyme kinetics or by Langmuir to describe the phenomenon of adsorption, an expression may be derived defining the equilibrium of the reaction :

$$\mathrm{HT} \rightleftarrows \mathrm{H} + \mathrm{T}$$

$$K = \frac{[\mathrm{H}]\,[\mathrm{T}]}{[\mathrm{HT}]} = [\mathrm{H}]_{\frac{1}{2}}$$

where [H] is the molar concentration of hormone in solution; $[\mathrm{H}]_{\frac{1}{2}}$ is that concentration required to produce half of maximal stimulation; and [T] and [HT] are the concentrations of unoccupied and occupied sites on the target organ. By a transformation similar to that of

Lineweaver and Burk, an expression is reached :

$$[\mathrm{H}]/[\mathrm{HT}] = K/Q + [\mathrm{H}]/Q$$

wherein $Q = [\mathrm{T}] + [\mathrm{HT}]$ and is a measure of the total concentration of target organ sites where hormone may reside. If the response to hormone in solution is, under given circumstances, proportional to the concentration of HT, target sites occupied by hormone, a straight line should result when hormone concentration divided by response is plotted against hormone concentration.

Entirely satisfactory data for testing of this hypothesis have not been found in the literature. Applications of these equations to certain published data have, however been considered encouraging. In a qualitative sense, the foregoing argument accounts for the fact that whereas, at low concentrations, the responses to many hormones are proportional to concentration, maximal responses have been reported to many hormones. Increases in hormone concentration beyond those required to elicit maximal response are without further effect.

Analysis of hormone action in these terms will have to await accumulation of suitable data in which a pure response to a hormone is measured in a relatively uncomplicated system *in vitro* over a wide range of known hormone concentrations. Should it prove valid, such analysis may permit comparisons of various members of a family of related hormones, as well as classification of hormone antagonists as 'competitive' or 'non-competitive'. It is offered here as a possible direction of development in the area of experimental endocrinology.

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# The mechanism of action of growth hormone

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(*Received* 19 *September* 1955)

Professor Sutherland has pointed out how important it is to correlate the results of *in vitro* studies with hormones with those produced by administration of the hormone to an intact animal. Only, in this way, can the physiological implications of *in vitro* effects of hormone be defined. One problem relating to such a correlation is that not infrequently species differences are noted with *in vivo* studies which are absent from *in vitro* experiments. Furthermore a hormone injected *in vivo* may have an effect on a tissue subsequently studied *in vitro* which is lacking when the hormone itself is added directly *in vitro* to the isolated tissue. These differences are particularly troublesome in studies with anterior pituitary hormones which have interested us for some years and a complete explanation of these at the molecular level is not yet available.

Differences between *in vivo* and *in vitro* studies may be explained in several ways. Thus the physical or chemical form of the hormone which influences the cellular reaction may be different from that which we extract from the parent gland and inject into the test animal. Furthermore the action of a hormone after injection may be mediated, in part, through any influence which it may have upon the rate of secretion of another hormone or other metabolically active substances. One or both of these influences may be lacking in *in vitro* studies.

An example of this type of complexity is provided by growth hormone which can exhibit growth promoting or diabetogenic activities under suitable experimental conditions. The most likely explanation of this difference of action is that the anabolic (growth promoting) action of growth hormone is dependent upon the availability of insulin in the organism. Thus if a deficiency of insulin is induced by growth hormone, relative to the needs of the organism at the time, then its action is diabetogenic rather than nitrogen retaining or growth stimulating.

The possibility that growth hormone stimulates the secretion of insulin by the pancreatic islets is one that has long been in mind. Recently at Cambridge, Dr. Randle has obtained further evidence upon this point by studies of the insulin activity of plasma from animals treated with growth hormone. He has used an *in vitro* method of insulin assay, first described by Groen and his collaborators, and based upon the glucose uptake of the isolated rat diaphragm. Randle (1) has obtained evidence suggesting that the administration of growth hormone to an intact cat leads to an enhanced rate of insulin secretion by the pancreatic islets. As a consequence, even when temporary growth-hormone diabetes has been induced in the cat, the plasma insulin activity may be well above normal. Randle finds also that hypophysectomy in the rat is followed by a decline in the level of insulin activity in the plasma and that this change may be prevented by treatment with growth hormone (1). In the analogous clinical conditions, acromegaly and hypopituitarism, Randle finds similar changes in the level of plasma insulin activity, the activity being enhanced in acromegaly and diminished in hypopituitarism (2, 3). However, in the intact rat, growth hormone does not appear to influence the level of insulin in the plasma (1). This does not necessarily mean that growth hormone does not enhance the rate of insulin secretion by the pancreatic islets of the rat, for growth hormone might influence both the rate of insulin secretion and utilization in the rat without altering the plasma level of insulin.

It is of interest that the action of insulin seems to be concerned in so many different mechanisms of metabolic control and it is unfortunate that despite the vast amount of research that has been carried out during the past

30 years, there is still no general agreement as to whether insulin acts by promoting the entry of glucose into the cells through a specific effect on glucose transport across the cell membrane, or by activation of the hexokinase system or by some other action.

The possibility that growth hormone is itself transformed in the body into another substance, possibly a lipoprotein, which exerts the anti-insulin activity possessed by growth hormone *in vivo* (but not *in vitro*) is a further complication in the elucidation of the mechanism of action of growth hormone by studies with *in vitro* systems. Furthermore, there is evidence now accumulating that some hormones, including insulin, may exist in plasma in a bound form in which fully physiological activity is not retained. Such a phenomenon might explain some of the discrepancies between estimations of the insulin activity of blood plasma by different methods of assay.

Not only do we find variations from one species to another in the response to hormones *in vivo*, but in some instances there appear to be surprising differences in the effects or different organs containing apparently similar tissues in the same animal. For instance, Greenbaum and Young (4) studied the distribution of protein among the muscles of rats treated with purified growth hormone and found that it was deposited in all the tissues examined, with the exception of the heart and the soleus muscle, but that the rate at which the individual muscles grew was very different. Pectoralis major, masseter, quadriceps, acromiotrapezius, deep spinal muscle, supraspinatus and the diaphragm grew significantly faster than the body as a whole, but no general biological picture could be drawn from the distribution of the muscles which grew most in comparison with those that grew least. It was found, however, that during inanition those tissues which gained protein most rapidly under the influence of growth hormone were those which lost it most rapidly under inanition, the masseter muscle being the exception in this connexion. Gray and Young (5) investigated the composition of the protein of the quadriceps muscle of rats which had been treated with growth hormone for 15 days. No differences were found, as the result of the treatment with growth hormone, in the proportions of the sarcoplasmic protein, myofibrillar protein and collagen-elastin fraction. The two components of muscle with adenosinetriphosphatase (ATPase) activity were investigated, that is, the ATPase activity of the myofibrils and the sarcoplasmic granules. In the rats which had been treated with growth hormone there was a significant diminution in the amount of myofibrillar ATPase per gram of fresh tissue. This finding might be correlated with the observation of Bigland and Jehring (6) who showed that although the weight of the quadriceps muscle of the rat may increase by 20-30 % as the result of treatment with growth hormone, the capcity of the muscle to develop tension does not simultaneously rise and when expressed in terms of tension developed per gram of muscle may actually diminish. It might be concluded that some or all of the material deposited in muscle under the influence of growth hormone is not effectively contractile. More recently, Korner and Young (7) have investigated the influence of the anabolic androgen methylandrostenediol on muscle composition, with results essentially similar to those of Gray and Young (5). The possibility must be considered that growth hormone, or a substance or substances liberated into the blood stream as the result of the action of growth hormone, facilitates the deposition of protein in muscle, but that other factors are concerned in the fashioning of contractile material from the deposited protein. At the present time the nature of these factors is quite unknown, but one possibility is that insulin is again implicated.

Despite all these complications it is of interest that recently Gray (8) has been able to show that the addition of growth hormone to diaphragm *in vitro* causes a significant uptake of radioactive glycine by the tissue. This action of growth hormone appears not to be mediated through the secretion of insulin, although it could depend upon the presence in the tissues of bound insulin which might be essential for this type of action.

Although the results of the *in vivo* administration of growth hormone are extremely complex and it may be sometime before we are able to duplicate, *in vitro*, effects produced directly and indirectly by growth hormone, a positive result of any sort is of significance at the present time, and it is clear that we should not hesitate to investigate the simplest system unless it is absolutely necessary to choose a more complex one. Unfortunately, with growth hormone the systems we have to study are undoubtedly complex, and although it will be sometime before the problem is solved we may hope that, as with glucagon and adrenaline, so with growth hormone, we shall be able to give some explanation of the protein effects of this perplexing protein in terms of an influence upon enzyme systems.

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# Some new thyroid-like compounds

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(*Received* 19 *September* 1955)

Until recently, the well known stimulation of metabolism *in vivo* by the thyroid hormone thyroxine has not been satisfactorily reproduced on *in vitro* systems (1); however, in the last few years work from the laboratories of Lardy (2) and of Lipmann (5) has shown that both thyroxine and triiodothyronine can effect uncoupling of oxidative phosphorylation in mitochondria. This reaction is not specific for the thyroid hormones, and compounds such as dinitrophenol and butyl-3,5-diiodo-4-hydroxybenzoate are even more efficient uncoupling agents than is thyroxine. Nevertheless a theory has been put forward that the mechanism of action of thyroid hormones is mediated through this uncoupling effect.

For some time Thibault has been searching for the peripherally active form of the thyroid hormone, and has lately reinvestigated the effects of thyroxine and of triiodothyronine on the respiration of tissue slices *in vitro*. Neither compound had any immediate effect, though they did produce some stimulation after a prolonged incubation with the slices at a low temperature. This had led Thibault to look for other compounds which might act without a latent period. Two such compounds have been found : the acetic acid analogues of thyroxine and triiodothyronine; both these compounds in low concentration produce maximal stimulation of oxygen consumption of kidney and liver slices from thyroidectomized rats in tyrode buffer within 15 minutes, after which the effect decreases and disappears after 90 minutes (3). Until now, no other thyroxine-like compound has shown this rapid action.

Another *in vitro* action of tetra- and triiodothyroacetic acids has been studied, by Heimberg and Park in America and by Isaacs and Pitt-Rivers in England (4), namely their effect on aerobic and anaerobic glycolysis in ascites tumour cells. Using cells from different tumours (Ehrlich and Sarcoma, 37) it has been found that marked stimulation of glycolysis occurs in the presence of triiodothyroacetic acid, though the concentrations required to produce maximal stimulation are much higher than those required in Thibault's tissue slice experiments. Triidothyronine has no stimulatory action of aerobic glycolysis in ascites tumour cells *in vitro* in concentration ranging from $3 \times 10^{-4}$ to $10^{-6}$ M; however, the effect is not specific to the thyroacetic acids : dinitrophenol gives a very marked effect (300 % rise in $CO_2$ produced aerobically) though at a high concentration, and 3,5-diiodo-4-hydroxyphenylacetic acid also has a slight effect.

It is possible, as suggested by Hoch and Lipmann (5) that the thyroacetic acids penetrate cells under the artificial conditions present in *in vitro* experiments much more rapidly than do thyroxine and triiodothyronine; this raises the question, how do the thyroid hormones reach their targets *in vivo* ? Are conditions then such that cell permeability is greatly increased, or is it possible that the hormones are converted to derivatives which can cross the cell membrane more easily ? At present there is no answer to this problem.

Both tetraiodothyroacetic acid and triiodothyroacetic acid can reproduce the effects of the thyroid hormones in the whole animal. Both have about one half of the potency of L-thyroxine in reversing thiouracil induced goiter in rats (6). The triiodo acid can restore the altered plumage of thyroidectomized birds to normal (7) and can stimulate growth in thyroidectomized rats (8); it also stimulates oxygen consumption in rats when given in repeated doses. Preliminary experiments by Thibault have shown that a single dose of the tetraiodoacetic acid produces a much more transient rise in the metabolic rate of thyroidectomized rats than is obtained with thyroxine or triiodothyronine; the latter compounds, after a latent period of 8-10 hours produce an effect which lasts for several days; tetraiodothyroacetic acid produces a metabolic stimulation within 2 hours, and the effect has greatly diminished or disappeared after 4 hours (3).

In the myxoedematous human subject, it has not yet been possible to produce an immediate effect with single doses of 5 mg. (9) or even 15 mg. (10) of triiodothyroacetic acid, but a large dose, *i.e.* 6 mg. per day, given orally in 4 hourly 1 mg. doses, does raise the metabolic rate in myxoedema (9). At the same time the usual weight loss and fall in serum cholesterol are observed.

There appear to be some quantitative differences in the actions of triiodothyronine and triiodothyroacetic acid on different metabolic functions in the human; triiodothyronine affects water diuresis, serum cholesterol and metabolic rate in the myxoedema patient concurrently (11) when given in small doses, and therefore reproduces the effects of thyroxine or of desiccated thyroid. If triiodothyroacetic acid is given in small doses, weight loss, a fall in serum cholesterol and clinical improvement have been demonstrated in two myxoedematous subjects, unaccompanied by any significant change in the metabolic rate or pulse rate (12). This phenomenon merits further investigation.

Another *in vivo* effect of triiodothyroacetic acid relating to allergic reactions will be briefly described. Tuberculin sensitivity can be induced in certain species such as man and guinea pigs by injections of B.C.G. vaccine, and Long and his colleagues (13) have studied certain factors influencing this sensitivity. Their work has shown that enhanced sensitivity can be induced in guinea pigs with insulin or by stimulating insulin production by prolonged treatment with thyroxine. After partial pancreatectomy, thyroxine is without effect. Conversely, diabetogenic substances such as alloxan, cortisone and growth hormone depress tuberculin sensitivity in susceptible species; it therefore appears that this type of sensitivity may be related to insulin output.

Long has suggested that a rapidly acting thyroid-like compound should be able to reduce tuberculin sensitivity by producing a thyroid diabetes, but neither thyroxine nor triiodothyronine had any immediate effect (however triiodothyroacetic acid given 1 hour before tuberculin had a marked desensitising action) (14). An experiment was devised whereby it was hoped to demonstrate directly that triiodothyroacetic acid acted by depleting the pancreas of insulin. Groups of guinea pigs were given triiodothyroacetic acid from one to five hours before the dose of tuberculin, and blood sugar determinations were done, at the time of tuberculin administration. At all time intervals, desensitization was observed, but there was no rise in blood sugar at any time. There is therefore at present no explanation for the desensitizing action of triiodothyroacetic acid.

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# Genetic function and molecular structure of DNA

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(*Received* 11 *June* 1955)

Just eleven years ago, Avery, MacLeod and McCarty (1) published their studies upon the chemical nature of the factor responsible for the induction of capsular transformations in pneumococcus. Over the past years, their paper has been one of the most often cited in biochemical and biological literature. Let us recall for a moment why this is so. Induced transformation, discovered by Griffith in 1928 (2), consisted in conferring upon unencapsulated pneumococci the ability to synthesize a capsule composed of a specific polysaccharide, by bringing the bacteria in contact with material obtained from encapsulated pneumococci. The phenomenon was recognized by Avery and others as probably the first established instance in which a hereditary property of an organism could be permanently and specifically modified. The excitement aroused by the 1944 paper of Avery and his coworkers was due to the discovery that the inducing agent seemed, on the basis of chemical and biochemical evidence, to be composed solely of desoxyribonucleic acid, a substance which had never before been suspected of possessing a complex chemical structure nor endowed with such highly specific biological function. Very largely under the impetus of this single publication, biochemists have again become actively interested in nucleic acids, substances which had been virtually neglected since the publication of the tetranucleotide theory of their structure. The past eight years of biochemical research upon these substances have brought about a revolution in our ideas concerning their basic composition. It is consequently of interest to return once more to the source of this burst of interest : to see what has become of our ideas about transforming agents as biological and chemical entities.

The studies of Avery, MacLeod and McCarty (3) indicated that desoxyribonucleic acid (DNA) is a substance endowed both with the capacity to cause the bacterium to synthesize a new, specific structure, unrelated chemically to the DNA itself, and with the capacity of causing the bacterium to reproduce more of the inducing agent. Both properties are those expected to be manifested by genes, and it was tempting to imagine that, at last, genes had been isolated in pure chemical form from one kind of cell, and reintroduced in active state into another closely related living cell. Biochemists and biologists voiced different reservations concerning such an hypothesis.

Biologists felt that capsular transformation was of unique occurrence and concerned such a highly specialized character — the capsule — that they were reluctant to generalize concerning its significance. Biochemists felt that the limitations of chemical and biochemical methods are such as to leave in doubt the true chemical identity of the transforming factor. Traces of other types of chemical substances, present in the highly purified DNA, might be responsible for the specificity of the active agent, the DNA serving essentially as a carrier.

It has proven easier to dissipate the doubts of the biologists, for doing so has entailed only the discovery of new kinds of transforming agents, concerned with very diverse cellular properties. Thus, today, we are in possession of agents affecting not only antigen syntheses, but also affecting fermentative abilities, antibiotic resistances of various sorts, resistance to an amino acid analogue, and determining the ability to oxidize lactic acid. Indeed, it has been the experience of those working on transformations that whenever a stable distinct character can be recognized, it has been possible to transform an appropriate test strain with respect to the character. It seems highly likely, therefore, that a very great number and variety of cell characters are under the genetic control of agents resembling the capsular agent in every respect except for being endowed with different specific activities. Lastly, transformation studies have been extended to other bacterial species, most notably to *Hemophilus influenzae*, by Alexander *et al.* (4), thus proving that this kind of phenomenon is not restricted to a single bacterial species.

The problem of establishing beyond any doubt the chemical identity of a transforming factor is a more difficult one. At the outset it was conceded that DNA must be an essential part of the capsular agent, yet could it be certain that the specificity of the agent was not due to small amounts of protein, for example, bound to the nucleic acid? The most sensitive analytical techniques failed to reveal protein in highly purified solutions of transforming factor (5). But the failure to find protein could not exclude its being present in trace amounts. Here the matter would necessarily remain

were it not for the fact that other types of evidence were brought to bear upon the question, by Hotchkiss (5), and in particular by Chargaff (6) and later by Wyatt (7). Chromatographic methods were developed for the quantitative separation of the bases liberated by hydrolysis from DNA obtained from unrelated species of organisms, and quantitative differences could be detected in the proportions of the bases. Thus, DNA, previously believed to be describable by a single chemical formula throughout the plant and animal kingdoms, was proven to be a substance which, like proteins, varied in composition from organism to organism. The fact that diversity of composition was shown to exist enormously increased the willingness to believe that the specificity of a transforming agent could reside solely in the arrangement of the atoms of the DNA molecules itself, because it became possible to imagine how different kinds of DNA molecules might be built up from purine and pyrimidine bases.

Experiments upon transforming factors have now amply confirmed the idea of McCarty and Avery (3), according to which a desoxyribonucleate isolated from a single kind of organism contains a multiplicity of transforming factors of different specificity. It is clear, therefore, that in order to define precisely in what way biological specificity depends upon chemical structure, methods must be developed by which a single transforming factor can be studied in pure state, separated from the unknown number of other factors with which it is normally mixed when extracted from the cell. We shall return presently to some attempts which have been made toward this end, but first of all let us consider what purely genetical experiments have been able to tell us concerning the structure of DNA, for it may well be that the genetical data will be a valuable guide in the execution of this task.

The very first transformation described, that for capsule synthesis, consisted in taking bacteria which had apparently lost the ability to synthesize a capsule, and by treating them with a DNA obtained from encapsulated bacteria, giving them back this synthetic capacity. The synthesis could be either of the same type as that lost by mutation, or to a different serological type. In the original experiments, the unencapsulated strain undergoing transformation was one which had never been observed to mutate spontaneously back to the encapsulated state. The obvious interpretation of the experiment was as follows : by mutation a bacterium lost its capsular agent, which, being an autoreproducing element, could not be regenerated. By transformation of the progeny of this bacterium, a capsular agent was put back into the cell. This image is compatible with the idea that the agent, lost by mutation, is a discrete particle present in the DNA extracted from the encapsulated bacteria. Notice that nothing can be said as to whether or not in the cell the DNA particle occupies a specific site. Were such a site to exist, according to this earliest picture of transformation it would be a site which can be perpetuated by the cell in the absence of the DNA particle with which it is normally associated in determining the capsule. In this picture, the heredity of the cell is dual : there is a part which can be perpetuated in the absence of DNA, and a whole element composed of site and DNA which causes the appearance of a highly differentiated character, the capsule.

In 1949, a completely different image of transformation could be constructed as a result of experiments on two distinct kinds of transformation (8, 9). The first line of experiments concerned a new transforming agent which determined a characteristic pattern of growth of non-encapsulated or rough pneumococci. Rough pneumococci growing in liquid culture form short chains of 4 to 8 diplococci, and growing on the surface of a solid medium form small colonies with distinct, smooth margins. A mutant form occurs which is distinguished from rough by the fact that it forms exceedingly long filaments of diplococci in liquid medium, and grows on solid media as a relatively large colony with uneven margins and nodular surface. Just as rough bacteria occasionally give rise to filamentous forms by spontaneous mutation, so do the filamentous forms revert by mutation to the non-filamentous state. Mutation is thus reciprocally possible between the two states. Here is a first reason for assuming that in this case, mutation of rough to the filamentous state does not involve a loss of an autoreproducing element, but rather its modification. Transformation studies fully confirm this point of view, for when nucleic acid isolated from rough, non-filamentous cells is placed in contact with filamentous pneumococci under appropriate conditions, about 0.1-1 % of these latter are transformed back to the non-filamentous state. The reverse experiment can also be performed ; *i.e.* rough pneumococci can be converted into filamentous forms under the action of DNA isolated from filamentous cells. Just as mutation back and forth between these two states is possible, so is transformation. These experiments strongly suggest that we are dealing with a genetic determinant capable of existing in two metastable forms, each recognizeable by a particular phenotypic expression. In this instance, transformation would seem to consist of the occasional substitution of the inducing determinant for the homologous determinant already possessed by the cell. Further experiments only strengthen this impression. Thus, if a rough bacterium is transformed to a filamentous one, and a nucleic acid prepared from the progeny of the transformed cell, only the filamentous determinant is found to be present in the DNA. The determinant of the non-filamentous condition, present in the rough bacteria prior to transformation, disappears as a result of this transformation.

A DNA isolated from a Type III encapsulated bacterium will not only transform rough bacteria to Type III smooth, but also will transform filamentous pneumococci into non filamentous ones. With one and the same DNA preparation, filamentous cells can be brought to the non-filamentous state, and then to the encapsulated state. The Type III bacterium thus produced can be demonstrated to possess both the non-filamentous agent, and that determining Type III capsule, just as does the original Type III strain from which the DNA was isolated. We see, then, that replacement of one agent by another occurs only when the two agents are closely related to each other ; that is, when one is a mutant form of the other, and when they determine a pair of alternative characters.

A second line of experimentation leads to the same conclusion. From time to time, encapsulated bacteria mutate to give rise to forms in which capsule secretion is reduced. These mutant forms can be shown propagate a mutated capsular agent. It is possible to transform the mutant bacteria back to normal with a DNA isolated from a normal strain. It is thus possible to observe what happens to the mutated agent as a consequence of this transformation. Here again it is found that transformation has resulted in the disappearance of the mutated agent in the progeny of the transformed cells, giving again the impression that the normal agent has been substituted in the place of the homologous, mutant agent which was originally derived from a normal agent by mutation. The fact that regulation occurs, so that the cell propagates only one of a pair of homologous agents in a permanent fashion, is a very strong argument in favor of the view that a specific site is involved, which can be occupied by only one or the other nucleic acid molecules.

Taking into account these considerations, the image one may now make of transformation takes on the following characteristics : as before, each specific agent can be visualized as a single particle in the DNA solution, but now the particle has acquired a specific locus in the bacterium. It is that site in the bacterium occupied by a homologous DNA particle. By homologous is meant an agent which is closely related to the inducing agent both in terms of structure and phylogeny. Defining the exact nature of this site is probably the most interesting and key problem which remains to be solved in order to understand how transforming factors are reproduced.

In this way, a very general image can be constructed concerning the nature of transformation; but purely biological studies lead also to very definite conclusions concerning the structure of the transforming factors themselves. The experiments now to be briefly summarized have led to a radical modification of ideas concerning the organization of the genetically active centers upon the DNA particles. Whereas at the outset there was no need to suppose that a single particle was concerned with more than one genetic function, today there is evidence which strongly suggests that this is too simple a picture. Let us turn now rapidly to three lines of evidence bearing upon this question.

The first indication of the complexity of the structure of a DNA molecule came from a study of a series of mutated Type III capsular agents (10). As has been mentioned above, in the course of its propagation in the bacterium, the Type III agent occasionally undergoes mutation, giving rise to agents having modified properties. The modifications thus far observed have all been quantitative, resulting in a decreased secretion of polysaccharide by the bacteria, without serological specificity of the synthesis being altered. One class of such mutations results in a very drastic reduction of secretion, while a second results in a less marked decrease. Normal Type III bacteria can be readily distinguished from either mutant variety, and the two sorts of mutants from each other, by simple inspection of colonies produced by the three kinds of bacteria. They can, of course, also be distinguished on the basis of agglutination reactions, these being affected both by the amount of soluble polysaccharide poured out into the medium, and the extent to which the cell surface is covered by the polysaccharide. Pneumococci propagating the mutant agent which induces the smallest secretion of polysaccharide can be readily transformed. It is thus possible to confront within a single coccus any two capsular agents of the series of mutated agents. In the studies which have been published, four mutants of independent origin of the class which secretes very little polysaccharide, and one in which secretion is only slightly diminished were thus confronted. Briefly, the results were as follows : a bacterium containing any one of the first four mutant agents, treated with a DNA obtained from the mutant in which secretion is only slightly reduced, gives rise to two sorts of transformed cells, their probability of occurring being approximately equal. On the one hand, transformations are found which correspond to the origin of the inducing agent; that is, to the mutant condition in which appreciable amounts of polysaccharide are secreted. On the other hand, transformations are observed as a result of which the bacteria acquire perfectly normal capsule synthesis. In these latter, it can be shown that not only is capsule synthesis restored, but also that the bacteria contain a normal Type III agent. Furthermore, neither mutant agent is present any longer. It is as though this second sort of transformation, given the name of allogenic transformation, has occurred as a result of some kind of interaction of the two mutant agents which gives rise to a normal one.

When this type of experiment is extended, so as to confront pairwise the various mutated agents, even though they appear to be identical in properties as judged from the phenotypes of the bacteria propagating them, allogenic transformations usually occur. In some instances, allogenic transformation leads to complete restoration of capsule synthesis, while in others it is only partial. In every case, the restoration obtained reflects an entirely parallel restoration of the capsular transforming agent itself. Furthermore, the result observed is specific for each pair of confronted agents. It is evident that the kind of allogenic transformation observed reflects the specific properties of the mutated agents involved.

A rather simple scheme permits one to visualize these results; it can be supposed that the capsular agent is composed of subunits, capable of mutating independently, and of undergoing recombination when two different agents are confronted within a bacterium. When recombination leads to complete restoration of the agent and the capsule, the mutated agents participating in the recombination can be visualized as possessing different mutated subunits. (See figure 1*a*). When, however, only a partial normalization can be obtained, the pair of mutated agents undergoing recombination may be supposed to possess in common a mutated region (See fig. 1*b*).

According to this picture, allogenic transformations giving rise to a partial restoration of the agent, as shown in Figure 1*b*, should occur much less frequently than

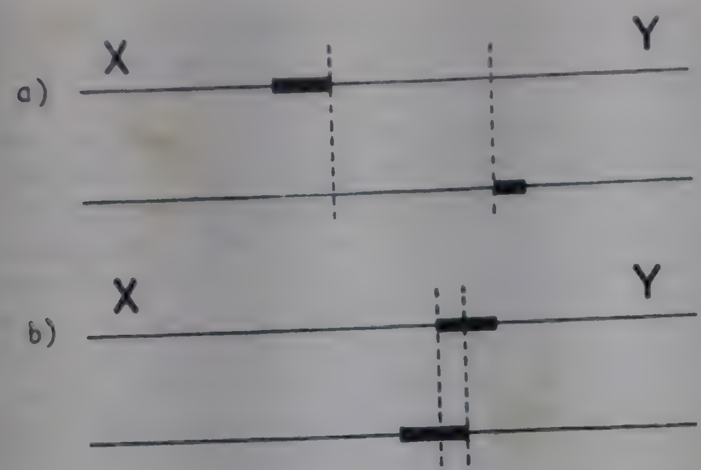


Fig. 1. — Diagram showing how recombination of between two mutant capsular agents can lead to restoration of a normal agent. Dark thick regions represent the areas altered by mutation. Dotted lines delimit the regions within which recombination must occur to eliminate the mutated areas. a) recombination restoring normal function. b) recombination in which only partial restoration occurs.

those giving rise to complete restoration, for they require the recombination to occur within a very restricted region. This, in fact, is what one observes. Furthermore, an agent arising from such a recombination should be unable to give allogenic transformations when tested on bacteria propagating either one of the two agents involved in its formation, though it should give simple transformation corresponding to its specificity, when acting on either. Lastly, the new agent should again give allogenic transformations when confronted with a different mutated agent. All of these expectations are verified when the experiments are performed.

We arrive thus, at an image of the DNA particle responsible for determining the Type III capsule as a particle composed of an unknown number of subunits which enjoy a degree of autonomy as expressed by their being able to undergo mutation independently of each other, and being able to recombine. Insofar as these experiments go, it is not possible to say whether or not the subunits have differentiated physiological function. While their mutation does not involve an alteration of the specificity of the polysaccharide secreted, it cannot be excluded that they represent loci controlling different steps in the synthesis of the complex polysaccharide of the capsule.

Critical information bearing on the problem of the extent to which subunits of a DNA particle are differentiated would seem to be provided by the experiments of Leidy, Hahn and Alexander (11) on the one hand, and Hotchkiss and Marmur (12) on the other. The first authors performed a series of experiments with capsular transforming factors isolated from *Hemophilus influenzae*. Three different serological types are concerned : types *a*, *b* and *d*. In a first group of experiments, bacteria of type *a* were treated with transforming agent type *b*, and there resulted the formation of bacteria synthesizing both *a* and *b* antigens. As would be expected, the *ab* bacteria possess also both *a* and *b* transforming agents, but the unexpected feature of this study was the discovery that transforming DNA prepared from *ab* cells confers upon an appropriate test strain both *a* and *b* antigens simultaneously, with what must be presumed to be a high frequency. In contrast, a mixture of *a* nucleic acid with *b* was never found to confer both antigenic synthesis upon a single cell, from which it may be supposed that under the conditions of study, the acquisition of two separate transforming factors is too rare to detect. The authors conclude that in the *ab* cells, both factors are linked to a single particle.

In a second group of experiments, unencapsulated *Hemophilus* cells, derived originally from type *b*, were treated with a transforming DNA prepared from *a* bacteria. A rare transformation was obtained from which, again, *ab* cells were formed. As before, the properties of the transforming extract were such as to indicate that *a* and *b* factors were structurally linked. The special aspect of this experiment is that the *b* antigen, presumably latent in the unencapsulated cell, reappears as a result of the transformation with the *a* factor.

The third group of experiments upon *Hemophilus* involve a new type, *d*. A transforming extract of a *d* strain was placed in contact with an *ab* strain, the latter being an *ab* strain in which the intensity of antigen synthesis has been diminished as a result of a spontaneous mutation. The transformation produced *ad* cells, characterized by a normal amount of capsule secretion. The transformation resulted in the formation of an agent in which *a* and *d* now seemed structurally associated, while all trace of the *b* agent and antigen had disappeared.

The ensemble of the results upon these capsular agents can be interpreted by supposing that in this species, too, the capsular agent has a complex-structure ; that in the course of evolution, mutations of the subunits have occurred which have resulted in qualitative as well as quantitative differences in the polysaccharide secreted, thus establishing the different serological types ; and that in the experiments described, we are observing recombination between the subunits of the DNA molecule determining capsular synthesis. It suffices to explain the results of Leidy, Hahn and Alexander to postulate four subunits : two determining specificity, and two suppressing the amount of capsule secreted. The *d* agent may be considered to be an allelic form of the *b* agent, for the cell seems to propagate only one, or the other, but not both at the same time. The relative positions of these four subunits are diagrammed in figure 2. In the first group of experiments described, recombination leads to the association of *a* and *b* upon the same DNA fiber ; in the second group of experiments, recombination is leading to the elimination of a suppressor closely linked to *b*, and the association of *a* and *b* on the same fiber. The suppressor has been drawn as situated close to *b* because the transformation is rare. In the third series of experiments, two alternate positions of the suppressor are indicated, for the results require that virtually every incorporation of *d* be accompanied by the elimination of the suppressor, a condition which is fullfilled with the suppressor in either indicated position. Other schemes to explain these results could, of course, be devised. The above is one of the simpler.

If the interpretation of these results is correct, the conclusion to be drawn is that the subunits of a DNA particle may concern differentiated functions.

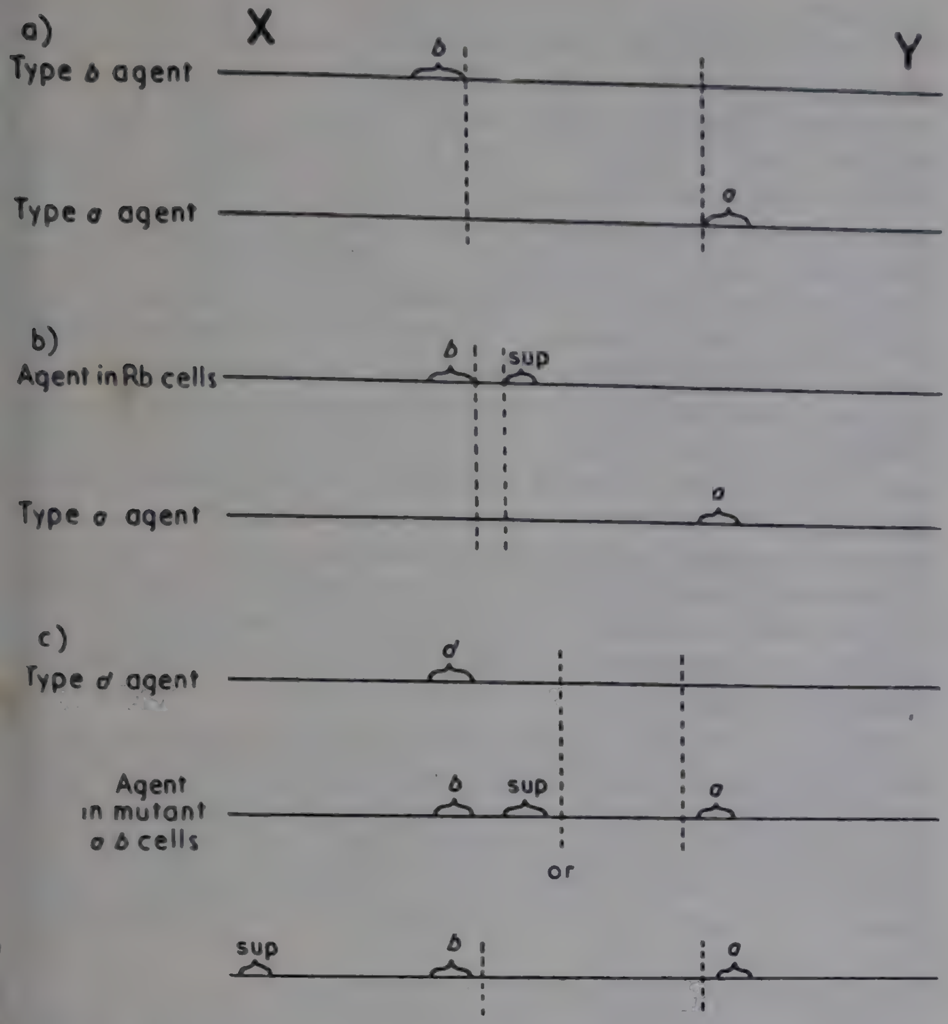


Fig. 2. — Diagram showing how recombination can explain the results of Leidy, Hahn and Alexander. Four differentiated regions are postulated, two for specificity of capsule synthesis, and two for suppression of polysaccharide secretion. For further details see text.

This impression is reinforced by the experiments of Hotchkiss and Marmur (12) working with transforming agents conferring resistance to streptomycin, penicillin and sulfanilamide, and an agent conferring upon the cell the ability to ferment mannitol. The authors describe experiments which suggest that both the mannitol factor and the streptomycin resistance factor are located on a single particle, with about three chances out of four of becoming separated through recombination during transformation. Thus, when transformations are induced with a DNA prepared from a strain containing the two presumably linked markers, both markers are acquired fairly frequently by the same bacterium. On the other hand, when a mixture is made of DNA isolated from two strains, each containing only one of the two markers, only very few double transformations ensue. The difference in results obtained under these two sets of conditions strongly suggests that both agents are located upon a single particle in the doubly marked bacterium.

Hotchkiss and Marmur present quantitative data on the relative frequencies of single and double transformations with the DNA preparations just mentioned. However, the exact meaning of their data is difficult to assess, owing to the fact that their calculations are based on several assumptions. The most critical of these is the assumption that the entire bacterial population is transformable at the moment the DNA is acting, an assumption which enters in a most critical way into the calculation of the relative frequencies of single and double hits. The method and theory of quantitative study of linkage remains to be worked out. Consequently, as in the case of the experiments of Leidy, Hahn and Alexander, those of Hotchkiss and Marmur are most convincingly in favor of linkage in those experiments comparing results in which two markers are introduced in separate DNA's prepared from singly marked strains on the one hand, and in single DNA from a doubly marked strain on the other.

If we accept the genetic evidence as adequate to demonstrate the complexity of a single DNA particle, we are thus led to a picture of the DNA fiber as differentiated along its length into functional subunits which possess a degree of independance of each other as expressed by mutability, discreteness of function, and recombinability. The picture which we now make of transformation is that of a process as a result of which segments of a DNA fiber are incorporated by the bacterium, taking the place of segments of a homologous fiber which the bacterium normally carries as part of its genetic substance. If two recognizeable, differentiated regions are situated closely enough together so as to be incorporated frequently in a single segment, we detect them as linked. If, on the other hand, two markers appear to be independently acquired, all we can say is that they are either on separate particles, or situated so far apart on the same fiber that the probability of a single incorporated segment including both is negligeable.

The emergence of such a picture as a result of genetic data alone brings us very directly to the problem of the chemical structure of DNA, and, indeed, to certain predictions concerning that structure. There remains, then, to discuss what these predictions are, and to see whether, already, chemical or physico-chemical evidence is accumulating in support of the predictions, drawn from genetic data alone.

One consequence of the image of the DNA molecule drawn above concerns chemical structure. If differentiation along the DNA fiber is sufficiently great that regions of the fiber determine different and distinct cell functions, considerable differentiation of chemical structure must also occur along the fiber. This being the case, it is evident that the study of the chemical basis of genetic specificity becomes a much more difficult problem. Depending on how many differentiated regions are to be found associated along one fiber, the application of fractionation methods may or not prove effective. Were a native DNA solution to be composed of ten varieties of fibers, each alone being the site of some hundred of differentiated regions, it is evident that the overall chemical composition of each fiber will be so similar that the chances of finding different affinities of the different fibers will be very small. Furthermore, if, actually, bacteria contain only one very giant DNA fiber, englobing all of the transforming factors of the cell, it is not fractionation of the native DNA which will reveal the chemical basis of specificity, but end group analysis.

In view of these considerations, it is interesting to consider very briefly the actual results currently being obtained by the application of fractionation methods to pneumococcal DNA. Two such methods have been described (13, 14). The data which I want to present were obtained by Brown and myself, using the method of Brown and Watson. This method consists in making an adsorption column out of kieselguhr to which histone has been adsorbed. DNA in dilute sodium chloride solution is retained by the column, and fractions can be eluted by washing the column with NaCl solutions of increasing molarity. The first material to come off of the column is composed of traces of RNA and any denatured DNA present in the preparation. No biological activity is found in it. It can be separated quantitatively from the bulk of the DNA because it forms a distinct peak in the elution diagram. Figure 3, showing

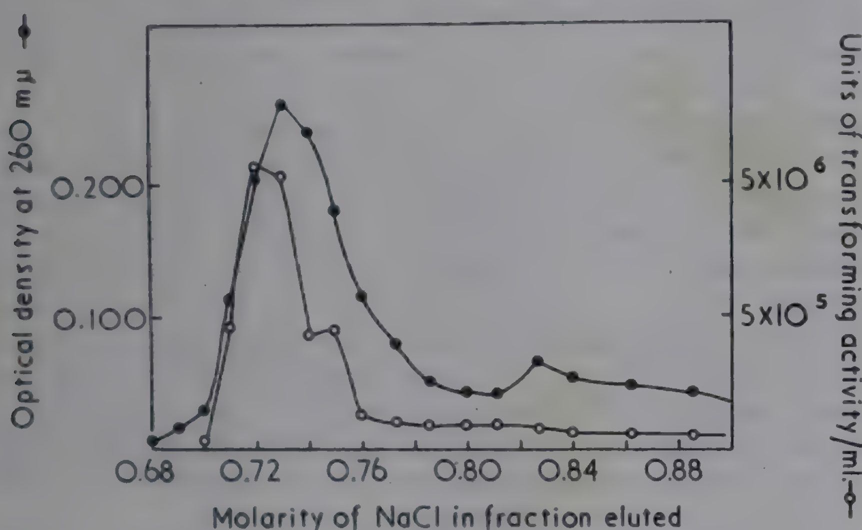


Fig. 3. — A fractionation of pneumococcal DNA, containing the streptomycin resistance factor, by the method of Brown and Watson (14).

a typical elution curve, does not include this first peak. The material which subsequently washes off of the column has the following characteristics : the fractions which are eluted first have the highest content of guanine and cytosine, and have the highest specific biological activity for each of the two transforming factors which have been studied by us. The two factors in question are the factor conferring a high degree of resistance to streptomycin, and that conferring resistance to canavanine, an arginine analogue. The elution of the first mentioned factor is shown in figure 3. In certain regions of the elution curve, the two factors seem to be present in significantly different amounts, notably in some fractions collected at the higher salt concentrations. It would, however, be a distinct error to suppose that any proper separation was yet achieved, in our work at least, for the general distribution of both factors throughout the curve is too similar to permit this illusion to be fostered. It seems more likely that the adsorption column was ineffective in these experiments or that the complexity of DNA structure is at the origin of the difficulty. Insofar as the first consideration is concerned, it should be pointed out that although fractionation of biological activity has not been achieved, a chemical fractionation has. How, then, can these contradictory results be reconciled? One possible way is to suppose that indeed the two factors are located on one, or two fibers composed of many differentiated subunits (if on only one fiber, the markers are far apart for the two factors do not appear to be linked) having essentially similar adsorption properties. It may be that although the DNA in the major elution curve is not denatured, the fibers have undergone some fragmentation during preparation. Were the fragmentation to entail a loss of guanine and cytosine, and the fragments to retain some biological activity, one might expect to obtain the observed elution curve. The material having the highest guanine content would be the most intact fibers, while that having low guanine would be fragments of fibers. One might expect to obtain with the guanine-poor material results approaching a separation of the two factors, if indeed this material represents small fragments of the native fibers. The smaller the fragment, the greater the chances of obtaining distinct differences in adsorption behavior.

Whether or not this proves to be the explanation of the fractionation results thus far obtained, it may very well be that the most logical approach to fractionation is to attempt to prepare biologically active fragment of DNA fibers, and subject the partially broken down material to fractionation, a task which we hope to undertake very soon.

A second structural consequence which emerges from the interpretation of the genetical data presented above is the following : it should be impossible to define the size of a single transforming factor by determining the size of the particles upon which it is located. In this connection, it is interesting to note the results of studies of the particles size of pneumococcal DNA by physico-chemical methods on the one hand, and by X-ray inactivation of biological activity, on the other. Using the light-scattering method, it has been estimated that the mean particle size of a pneumococcal desoxyribonucleate correspond to a molecular weight of $7 \times 10^6$ (15, 16). Upon the same solutions, X-ray inactivation curves indicate that the streptomycin resistance factor has a sensitive volume corresponding to a molecular weight not exceeding $7 \times 10^5$. (Only a maximum size can be assigned by the X-ray method, owing to the difficulty of excluding all inactivation due to indirect effects). There is, consequently, a discrepancy of at least ten-fold in the two sorts of measurements.

There are several possible reasons for this. In the first place, the light-scattering method provides only an estimate of average particle size, and if the solution is not homogeneous, in general very large particles have more weight in influencing the measurements than do smaller particles. It could be, therefore, that the discrepancy is due to inhomogeneity of size of the DNA particles, and that the streptomycin factor is found, or can be detected biologically, only on the smaller particles. However, a second possibility can be envisaged. If, indeed the streptomycin factor occupies only part of the length of a DNA fiber, it is conceivable that the sensitive volume measured by X-ray inactivation represents essentially the region within which a hit must occur for activity to be destroyed. If the energy travels only a

part of the length of the fiber, the sensitive volume will necessarily measure neither the size of the fiber nor the actual size of the region occupied by the transforming factor studied. The sensitive volume may represent, in fact, the average distance over which the energy of an ionization will travel in the molecule. Or, if the action of the ionization is to fragment the molecule, it may represent the minimum size of a fragment, containing the region for which the biological test is designed, which is still capable of being incorporated by the bacterium. This second possible explanation of the significance of the sensitive volume is somewhat complicated by the fact that inactivation curves demonstrate a definite heterogeneity of the solution; that is, at higher X-ray doses, as inactivation proceeds, more resistant particles are encountered. These resistant particles represent about 10 % of the total activity of the DNA solution. At present, it is difficult to state whether they are particles having about 1/6 the size of the material giving a molecular weight of $7 \times 10^6$, or whether, on the other hand, they indicate the presence of aggregates of DNA fibers requiring a large number of hits for their inactivation. Attempts have been made to cause their disappearance, by treating the DNA solutions with strong urea, to diminish intermolecular attraction, but no effects of the urea have been observed (17).

These various considerations serve to show the extreme complexity of the problem with which one is faced in determining the basic facts of the molecular structure of DNA. Not only has the extreme asymmetry of the DNA particle hampered the physical chemist at the outset, but now the genetic data suggest that the relationship between structure and function is more complicated than had been imagined : that these giant molecules are multifunctional, and probably differentiated along their long axis. Success in disclosing how chemical structure determines biological function may, therefore, depend entirely upon our being able to break up these fibers into small fragments still possessing detectable, specific biological activity.

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## The genetic significance of viral DNA

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(*Received* 24 *March* 1955)

At a biochemical congress a review subject is the better suited the more it appeals to both chemist and biologist. Let us first see whether a discussion of viral DNA satisfies this requirement.

The chemist essentially seeks knowledge about structure and interaction at the molecular level, and if he works in biology his tendency is to destroy the cells in order to isolate and study particular molecular species. To a biologist the idea of a cell-free system is most unnatural because he is interested primarily in the features which distinguish the living cell from an unorganized, homogeneous chemical system. In a few cases this gap between chemistry and biology almost disappears; thus, in recent years, chemists studying the properties and structure of DNA have worked in close contact with biologists, more precisely geneticists, studying the central role of DNA in controlling orderly reproduction. It is this fortunate situation which lends special significance to current studies of the transforming principle of bacteria and of the DNA of bacteriophages.

Chemical studies of purified DNA are most interesting to biologists when it is known that the information obtained relates to material which has retained the unique genetic properties of the DNA. The transforming principle of bacteria is a case in point : thus crystallized and biologically highly active preparations have yielded elegant X-ray diffraction patterns of the types used by Watson and Crick when developing their suggestive structural model of the DNA molecule (1). — In the case of the bacteriophage the situation is somewhat different : the

free virus particle consists of a core of phage DNA neatly parcelled up in a protein envelope; the DNA to protein ratio is close to unity. This condition is not favourable for diffraction studies of the native DNA, although « powder » diagrams which agree with the proposed model have been obtained from samples of viable phage; on the other hand, the extreme chemical and biological stability of the extracellular, or resting, phage particle makes it an ideal object for certain tracer experiments. The isotopes $^{32}P$ and $^{35}S$ are obvious choices since differential labelling of the DNA or the protein of the particle is automatically achieved. Free phage particles, uniformly labelled with $^{32}P$, have been used in experiments of several kinds, some of which I propose to use to illustrate aspects of phage work, which are concerned directly with the functions of the DNA.

Incorporated $^{32}P$ atoms can be used not only to trace DNA but also to probe its resistance to the damage caused by disintegration of $^{32}P$ atoms. Studies of the viability of phage particles which carry a considerable proportion of $^{32}P$ atoms in their DNA have revealed, first, that a single $^{32}P$ disintegration suffices to kill a phage particle, and second, that only 1 disintegration out of about 12 is effective (2). These conclusions were based on the kinetics of the killing curves and on the specific activities of the phage phosphorus, and were taken as an indication that only about 1/12 of the phage DNA was essential material and that any disintegration within this part caused death of the particle. Later experiments of the same kind showed that the fraction 1/12 reduces to 1/20 at very low temperature and that two quite unrelated phage strains give nearly identical values for the radiochemical efficiencies (3). It is therefore not possible, on this evidence, to decide whether all or only a fraction of the phage DNA is essential for reproduction. The relative insensitivity of a phage particle to the decay of incorporated $^{32}P$ may simply be a manifestation of the stability of the DNA molecule. The model of Watson and Crick in which two copies of the genetic code are contained in a highly stable double-helix, suggests that perhaps only such $^{32}P$ disintegrations as cause secondary damage would destroy the capacity of a molecule to form at least one sound replica of itself. It seems premature, however, on the basis of the model alone, to speculate further in this direction.

Another type of isotope experiment has subsequently been carried out in an attempt to decide whether the DNA of a phage particle constitutes a single, essential unit, or whether part of it is dispensable. Suppose that a small unit within the DNA were essential for reproduction, that it remained genetically intact and exchanged phosphorus with the corresponding units of new particles only, while the rest of the DNA did not participate directly in reproduction. The existence of such a unit might have been revealed by the following experiment: phage labelled uniformly with $^{32}P$ was allowed to reproduce in unlabelled bacteria, and the fraction of the label which reappeared in the DNA of the progeny was determined, this process was repeated using the progeny phage to infect new bacteria in order to see whether the $^{32}P$ transmitted during the first reproduction cycle might be located in an essential unit whose $^{32}P$ would be selectively transmitted during the second cycle. It was found that about 1/3 of the parental $^{32}P$ was transmitted to the progeny in both cycles of reproduction. Thus a differentiation of the phage DNA into essential and non-essential parts was not demonstrable (4).

Most of the experiments with resting or free phage, a few of which have now been described, were carried out before it was known just how decisive the role of DNA is in phage reproduction. This was revealed by Hershey and Chase (5) who used phage T2 labelled with $^{32}P$ or $^{35}S$ to provide the following important information: the protein of the phage particle behaves like a passive, protective shell around the DNA; this shell, including the tail by which the particle attaches itself to the sensitive bacterium, is left empty on the outside of the cell into which all the phage DNA passes through the hollow tail. Once the « injection » of the DNA has occurred the protein shell has played its part, and the course of infection is not affected by stripping the shell off, *e.g.* in a Waring blendor. This sequence of events has been constructed from the tracer experiments together with direct observations by electron microscopy (6).

The implications of these findings are numerous: on the one hand, completely new conclusions have been reached, while on the other hand, a palpable basis has been provided for genetic theories which, at the time of Hershey's discovery, had developed far ahead of chemical evidence.

The new aspects which have emerged are perhaps best put into perspective by pointing out some similarities and dissimilarities between the transforming principle and the DNA of bacteriophage: the chemical purity of the transforming principle is so well established that it may be taken for granted that transformation is effected by DNA molecules which carry the determinants of specific genetic traits. Hershey's results suggest that, similarly, phage DNA determines the genetic traits of the bacteriophage. Admittedly, the case for ascribing this function to DNA molecules is not so strong in the second as in the first instance, simply because bacteria cannot be infected by phage DNA which is freed artificially of protein. Attempts in this direction have not been pushed very far and, *a priori*, success seems unlikely. The problem is not, as in transformation, to have the cell accept a small piece of DNA carrying one or a few genetic determinants, but to get a very large DNA structure carrying a vast number of determinants, intact across the cell boundary. However satisfying it would be to ascertain that nothing essential apart from DNA enters the cell when it is infected with phage, proof is difficult, if not impossible to obtain, with the methods now available.

Therefore, our assumption that DNA molecules determine the genetic traits of the bacteriophage rests on the knowledge that DNA is capable of this function, which is provided by the transforming principle, and on Hershey's data which show that little, if any, protein enters the bacterial cell with the DNA of the phage particle.

Accepting this evidence as sufficient, we may ask whether studies of phage DNA have added new features to our concepts of the genetic function of the DNA molecule. The answer is undoubtedly yes. To this we may add

that most of the information which had not already been provided by the transforming principle, has been obtained by combining morphological, chemical and genetic evidence.

As pointed out previously, no indication of a subdivision of the phage DNA into functionally distinct portions has been obtained, despite rather intensive search. We shall therefore assume that all the phage DNA is genetic material which, in the cell, undergoes replication. The fact that this DNA passes through the tail of the phage particle makes it almost certain that it arrives in the bacterium as one or more long filaments. It has not been possible to observe this, but the injection process has been imitated by allowing phage particles to combine with isolated bits of the membrane of sensitive bacteria ; this combination causes the DNA of the phage to be expelled into the medium, and electron micrographs of such preparations show empty phage heads together with irregular bundles of thin threads of DNA (7). Similarly, phage DNA which is released by more artificial physical means appears as filaments measuring 20 A, or somewhat more, in diameter and more than 10 000 Å in total length (8). We assume, therefore, that phage DNA arrives in the cell as one or more enormously long threadlike molecules, and for structural as well as genetic reasons we believe that this is the form in which replication occurs. To avoid steric complications during replication, the long filaments, steadily increasing in number, probably coil and uncoil in the cell. At the moment, nothing is known about the molecular organization of the replication process.

What is known about this process comes from kinetic and genetic studies. The net result of the kinetic evidence is that, before any finished phage particles are formed in the cell, a pool of 50-100 phage equivalents of viral DNA is created. The existence of this pool was indicated by the observation that finished phage particles contain a mixture of early and late assimilated $^{32}P$ (9). Subsequently it was discovered that the DNA of the T2, T4 and T6 phages is distinct from that of the host cell in that it contains 5-hydroxy-methyl-cytosine instead of cytosine (10) ; this discovery has permitted studies of the formation of the DNA pool by direct chemical identification of the viral DNA (11).

Before this chemical evidence was available, the genetics of phage production had been studied intensively. The basic genetic experiment with phage consists of infecting individual bacteria with two or more particles carrying different genetic markers that can be identified by inspecting the lytic zones, or plaques, which phage particles form when put on an agar plate together with sensitive bacteria. In this way Doermann discovered that, prior to the appearance in the cells of the first complete phage particles, unidentified precursor particles exchange genetic material, with the result that new combinations of the genetic markers appear (12). These results together with the chemical evidence for the existence of a pool of viral DNA, obviously led to the assumption that genetic recombination occurs between elements in the DNA pool. This idea is important because it implies that the pool contains genetically fully differentiated DNA molecules and not just precursors of such molecules.

The fact that DNA replication and genetic recombination both occur with considerable efficiency during the same period raises the intriguing question : is there a causal relation between the two processes ? *viz.* does recombination occur only when two DNA molecules undergo replication in a synchronous manner on the same site in the cell ? An answer to this question may be sought when a solution has been found to the difficult steric problems involved in the replication of a structure like the DNA molecule (13).

The prototype of a genetic experiment with phage, described a moment ago, has been varied in many ways, and a special type of experiment which sets off the autonomy of the phage DNA is particularly interesting : a cell infected with the two closely-related phages T2 and T4 will, in addition to new T2 and T4 particles, produce some hybrids which are composed of a DNA core of, say, the T2 type and a protein shell and tail of the T4 type. The host specificity of such a hybrid is determined by the T4 type protein of the tail, and the hybrid can therefore adsorb on and inject its DNA into a bacterium resistant to phage T2 whose DNA is in this manner surreptitiously introduced into the cell. The significant feature of this experiment is that all the new particles formed in the cell are regular T2 phages. This means that the type-2 DNA has induced the production of the specific T2 protein which is not produced by the uninfected bacterium and for which the hybrid phage particle did not provide a model (14). This result is, I think, clear evidence that the material which entered the bacterium and initiated phage production carried the genetic determinants which — in the cell — directed the synthesis of the specific T2 protein. Incidentally, this conclusion is valid whether the injected material is pure DNA, as we have assumed, or a nucleoprotein.

It has been pointed out before (13, 15) and may be repeated : it is of the utmost importance for our concepts of genes and gene function that we have reached the point where it seems natural to identify the genetic determinants, or genes, with DNA molecules. These linearly differentiated molecules can well be imagined to serve as a tape conveying directions for a defined sequence of chemical actions, thus, in the case just described, directions for combining amino acids into that particular sequence which produces a molecule with the specific properties of the T2 protein. Just how the genetic notation of the DNA is translated into protein structure is unknown ; however, Gamow has already shown that the DNA model of Watson and Crick indicates a way in which the translation might possibly occur (16).

In order to arrive at these far-reaching conclusions it has been necessary to rely chiefly on the very thorough studies of phage T2. From the advanced point to which these studies have taken us it is desirable to turn and see whether our present ideas about the functions of phage DNA may shed light on observations made under special conditions or on other systems.

It was mentioned above that genetic recombination in phage T2 seems to occur at the time when the phage particle is nothing but a naked string of DNA. Another important feature of this genetic system is that recombination apparently occurs in a random manner among a

considerable number of similar DNA elements, each element exchanging parts with others several times before it is withdrawn from the genetic pool and fitted with its protein shell (17). Once this process of maturation is complete inside the cell, the particle seems to be as stable and genetically inert as a free phage.

It is an interesting but unsettled problem whether the process leading to genetic recombination is also responsible for other manifestations of interaction between phage particles and between phage and bacterium. First, let us consider the phenomenon of multiplicity reactivation (18), *i.e.* the fact that ultra-violet irradiated phage particles which are unable to multiply individually are readily reactivated when introduced together into the same cell. This startling phenomenon is particularly pronounced in phage T2 and related strains, which also show very efficient genetic recombination, these two effects were therefore initially ascribed to the same recombination mechanism. However, the simple and suggestive model developed to account quantitatively for multiplicity reactivation was shown to be inadequate (19), and the phenomenon, now lacking in appeal, was considered more or less as a special manifestation of ultra-violet irradiation.

The more general significance of multiplicity reactivation became obvious when it was demonstrated that inactivation by several types of radiation may be repaired through interaction between damaged particles. Thus, Weiglé and Bertani found that, under appropriate conditions, X-ray inactivated phage exhibit multiplicity reactivation (20), and Stent showed that the stability of $^{32}P$-labelled, immature phage particles is greatly increased as long as they can recombine with other immature particles (21). The latter experiment requires some explanation : bacteria were infected with phage, both components as well as the growth-medium being uniformly labelled with $^{32}P$, and at various times after infection samples were removed and stored in liquid nitrogen at — 196° C. ; the ability of the infected cells and of the free phage to form plaques when plated on agar was determined after storage in the cold for different lengths of time. The interesting result was that, due to $^{32}P$ decay during storage, phage particles formed in the labelled environment soon lost their viability, whereas cells with a fully developed DNA pool remained capable of producing at least one sound particle. This stability and the reactivation of ultra-violet and of X-ray inactivated phage are probably the results of a recombination process by which damaged parts are exchanged for sound ones. It is tempting to identify this process with that which causes genetic recombination, and at the moment it seems natural to adopt a unitarian view.

I should like to stress that until now we have discussed exclusively experiments carried out with the so-called virulent phages, *i.e.* phages which invariably destroy the bacterial cells they multiply in. It is therefore pertinent to ask whether our notions about the role of phage DNA in replication and recombination apply also to the vast class of temperate phages, *i.e.* phages which are capable of existing in stable symbiosis with their host cells. A bacterium which carries a phage in this way is called lysogenic, and it is generally accepted that the intracellular phage particles are incomplete ; in this state the carried phage is referred to as prophage. Studies of genetic recombination between lysogenic bacteria indicate that the prophage is in some way attached to the bacterial nucleus (22, 23), and ordinary phage experiments have shown that the temperate phage λ undergoes genetic recombination in much the same way as T2, though less efficiently (24). Multiplicity reactivation has been looked for but not demonstrated in temperate phages.

The results of the recombination experiments with phage λ suggest basic similarities between the genetic functions of the DNA of temperate and virulent phages. Recently, two examples of recombination under very special circumstances have been discovered in temperate phages. First, Bertani found that crosses between two bacterial strains which are lysogenic for two mutants of the same phage, give rise to a few lysogenic cells in which markers on the bacterial chromosomes, as well as prophage markers, are recombined (25). This result indicates that the attachment of the prophage to the bacterial nuclear apparatus is so intimate, and the two associated structures so much alike, that the prophage may behave, genetically, as part of the bacterial chromosome. Second, an effect which somewhat resembles multiplicity reactivation has been described by Weiglé (26) ; he discovered that ultra-violet irradiated λ phages which did not survive in normal cells could be reactivated when adsorbed onto ultra-violet irradiated bacteria. Among the progeny produced in such a system were a large number of different mutant phage particles. The interpretation given to these results is that, in the ultra-violet irradiated cell, damaged phage parts may be exchanged against undamaged and closely related parts derived from the bacterial nucleus. Again, this example points to an intimate relationship between the DNA of a temperate phage and that of the host cell.

In conclusion, it seems justifiable to express some optimism with regard to the future usefulness of the genetic concepts which have evolved, partly from chemical and structural analysis of the DNA molecule, and partly from biological experiments with the transforming principle of bacteria and with bacteriophage. The results of recent years' work in these fields show, I think, that a great step has been made towards an understanding of microbial genetics at the level of molecular structure.

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## Le rôle de l'acide ribonucléique dans la multiplication des virus

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(*Reçu le* 10 *août* 1955)

Les données tirées de l'étude des facteurs de transformation et du mécanisme de la multiplication des phages indiquent clairement que la spécificité de protéines peut être commandée par celle de molécules d'acide désoxyribonucléique. Mais il serait prématuré de généraliser exagérément cette notion et d'admettre que l'acide désoxyribonucléique est le seul facteur susceptible d'imposer à une protéine sa spécificité. Si l'acide désoxyribonucléique agissait seul, et directement, comme modèle dans la synthèse de toutes les protéines, nous devrions imaginer que le noyau cellulaire est le siège exclusif de cette synthèse, ou tout au moins de son stade final. Brachet et ses collaborateurs ont montré qu'il n'en était rien par leurs expériences sur les effets de l'énucléation (1). Le problème des facteurs responsables de l'édification de protéines spécifiques ne peut être examiné que dans son ensemble et il serait difficile de dissocier les données fournies à ce sujet par l'étude du rôle de l'acide désoxyribonucléique du noyau, et celles relatives à l'intervention très probable de l'acide ribonucléique, présent principalement dans le cytoplasme. Un examen critique de l'état de la question a été publié récemment par Brachet (2) et je n'ai pas à m'y arrêter au cours de cette brève intervention. Je n'y fais allusion que pour souligner l'intérêt qu'aurait une démonstration du rôle de l'acide ribonucléique dans la détermination de la spécificité des protéines aussi claire que celle fournie, dans le cas de l'acide désoxyribonucléique, par les recherches exposées dans les rapports de Mme Ephrussi et de Maaloe. Pareille démonstration n'existe pas à l'heure actuelle. Nous ne le savons que trop. Je voudrais essayer de montrer que nous n'en sommes peut-être pas aussi éloignés que nous pourrions le croire.

La composition de plusieurs virus de végétaux a été étudiée avec suffisamment de soin pour que nous puissions considérer comme très improbable qu'ils contiennent un autre acide nucléique que l'acide ribonucléique. Cet acide ribonucléique joue-t-il dans la multiplication des virus de végétaux un rôle comparable à celui de l'acide désoxyribonucléique dans celle des phages ?

Diverses modifications de la portion protéique du virus de la mosaïque du tabac ont pu être obtenues par substitution de groupes divers, fixation de résidus d'acides aminés supplémentaires, élimination d'acides aminés en bout de chaîne, sans que le pouvoir de multiplication des particules ainsi transformées, ni les caractères de leur descendance ne soient altérés. Par contre, la formaldéhyde, qui supprime réversiblement l'infectivité du virus de la mosaïque, n'agit pas par ses réactions avec la portion protéique du virus mais très vraisemblablement en se combinant avec des groupes aminés de l'acide ribonucléique (3). Il est apparu de plus que de nombreux analogues de purines et de pyrimidines étaient des inhibiteurs puissants de la synthèse du virus et que deux d'entre eux au moins, la 8-azaguanine et le thiouracile, s'incorporaient dans l'acide ribonucléique du virus sous la forme de nucléotides anormaux. Les particules de virus ainsi modifiées dans leur seule portion nucléique manifestent une diminution très notable de leur infectivité (4, 5, 6). Tous ces faits plaident en faveur de l'idée que l'acide ribonucléique doit jouer un rôle essentiel dans la multiplication du virus et qu'il constitue l'élément auquel une chimiothérapie rationnelle des viroses végétales doit s'attaquer.

L'importance de l'acide ribonucléique des virus de végétaux ressort aussi clairement du fait que les particules du *turnip yellow mosaic virus*, dépourvues d'acide ribonucléique mais identiques au virus à tout autre point de vue, ne sont pas infectieuses (7). De même, les particules fort semblables au virus de la mosaïque du tabac, qui peuvent être obtenues *in vitro* par polymérisation d'une protéine de faible poids moléculaire présente à côté du virus dans les plantes malades (antigènes solubles) sont exemptes d'acide nucléique et non infectieuses (8, 9, 10).

Compte tenu de ces données, la démonstration fournie par Markham que l'acide ribonucléique du *turnip yellow*

*mosaic virus* se trouve enfermé dans une molécule protéique et la découverte (11) que l'acide ribonucléique du virus de la mosaïque du tabac se présente comme une sorte de baguette centrale autour de laquelle viennent s'ordonner en une couche continue les molécules de protéines, suggerent d'évidents rapprochements entre les virus de végétaux et les phages.

Citons quelques résultats récents qui confirment pareille idée. En premier lieu, la vitesse avec laquelle des acides aminés marqués s'incorporent dans les petites molécules de l'antigène soluble du virus de la mosaïque du tabac est considérablement plus grande que leur vitesse d'incorporation dans les protéines normales ou le virus. Cette grande vitesse d'incorporation s'interprète aisément sur le plan quantitatif si nous admettons que l'antigène soluble du virus de la mosaïque du tabac est le précurseur de la portion protéique du virus (12). Nous devons donc admettre que la croissance du virus commence par l'accumulation de précurseurs protéiques dépourvus d'acide ribonucléique et que, tout comme dans le cas des phages, la virulence naît lorsque ces précurseurs protéiques et de l'acide nucléique, également synthétisé par la cellule, s'unissent en un édifice commun.

S'il en est ainsi, nous devions nous attendre à ce que le développement du virus de la mosaïque du tabac commence par une phase d'éclipse. Les travaux récents de Zech rendent son existence très vraisemblable (13, 14).

Enfin, la ribonucléase infiltrée dans une feuille de tabac, durant les quelques heures qui suivent immédiatement l'infection, empêche tout développement du virus. Cette même infiltration opérée plus tard est au contraire totalement dépourvue d'effet (15). Comme la ribonucléase est sans action sur l'acide ribonucléique du virus aussi longtemps que celui-ci est protégé par sa gaîne protéique, il est tentant d'imaginer qu'au cours d'une phase initiale de l'infection l'acide ribonucléique est expulsé de cette gaîne comme l'est l'acide désoxyribonucléique des phages. Ajoutons que la ribonucléase agit bien en tant qu'enzyme puisqu'une inactivation par $H_2O_2$ supprime son action inhibitrice sur la croissance du virus.

Si notre interprétation de tous ces faits est exacte, il devient difficile d'échapper à l'idée que l'acide ribonucléique des virus de végétaux joue le rôle de modèle dans la synthèse des protéines spécifiques du virus tout comme nous avons été amenés à en faire l'hypothèse dans le cas de l'acide désoxyribonucléique des phages.

Un second point sur lequel je voudrais attirer l'attention concerne le mode d'action de l'acide désoxyribonucléique des phages dans la synthèse de la portion protéique de ces virus. L'idée a été exprimée de divers côtés déjà que la molécule d'acide désoxyribonucléique, injectée dans la bactérie, agit comme un modèle sur lequel vient s'édifier la protéine spécifique correspondante et que l'acide ribonucléique n'interviendrait donc pas dans cette synthèse. Pareille hypothèse repose en partie sur des observations de Cohen suivant lesquelles, dès le moment de l'infection, la quantité d'acide ribonucléique cesse d'augmenter, cette substance manifestant de plus une très grande inertie métabolique comme en témoigne l'absence d'incorporation appréciable de phosphate marqué. Hershey (16), dès 1953, a cependant signalé avoir constaté une incorporation de phosphate marqué dans l'acide ribonucléique après l'infection et insisté dans une revue récente sur le fait que l'intervention de l'acide ribonucléique dans la multiplication des phages n'est nullement exclue (17).

Des expériences effectuées au cours de ces derniers mois pourraient fournir un argument nouveau à pareille idée. Ainsi que Groth (18) l'a montré récemment, la ribonucléase inhibe l'incorporation d'acides aminés marqués dans les protéines de *Bacillus megatherium* et la croissance de cette bactérie. Nous avons profité de cette observation pour faire agir de la ribonucléase sur une souche lysogène de cette bactérie immédiatement après induction par les rayons ultra-violets de la production de phages. A des concentrations faibles la ribonucléase n'inhibe pas la lyse mais provoque une diminution massive du nombre de particules de phage libérées (19). Puisque toutes les cellules se lysent, ce n'est pas la transformation du prophage en élément capable de se multiplier qui est inhibée, mais bien, vraisemblablement, la synthèse des constituants protéiques du phage. Pareille recherche devra être poursuivie et son résultat précisé. S'il apparaissait que des molécules spécifiques d'acide ribonucléique apparues sous l'influence de l'acide désoxyribonucléique injecté dans la bactérie jouent un rôle dans la synthèse des protéines du phage, le mécanisme de la multiplication des phages serait un modèle particulièrement utile dans l'analyse des facteurs de la synthèse des protéines au sein de la cellule, facteurs parmi lesquels il paraît difficile de ne pas placer l'acide ribonucléique aussi bien que l'acide désoxyribonucléique.

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# Incorporation of amino-acids by disrupted staphylococci : replacement of ribonucleic acid by its digestion products

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(*Received* 7 *April* 1955)

The investigations of Caspersson (1) and Brachet (2) first indicated a correlation between the nucleic acid content of cells and their rate of growth. The correlation has since been confirmed by numerous workers studying a variety of tissues and, in the case of *Staphylococcus aureus* where it is possible to alter the nucleic acid content by incubation with and without antibiotics under various conditions, it has been possible to show that a change in the nucleic acid content of the cells is followed by a corresponding change in the rate at which those cells are able to synthesise protein (3). The first direct demonstration that nucleic acid is essential for protein synthesis has now been obtained with disrupted staphylococci where removal of nucleic acid results in cessation of protein synthesis, and replacement of the extracted nucleic acid enables the cell fragments to resume specific enzyme synthesis (4).

Numerous examples of bacterial transformation have now been described and, in a number of cases, the specific transforming principle has been shown to consist of deoxyribonucleic acid (DNA), (5, 6, 7). The alteration in bacterial metabolism following infection with bacteriophage appears to be due to the passage of DNA from the bacteriophage into the cytoplasm of the host cell (8). The synthesis of adaptive enzymes in bacteria and yeasts can be inhibited by the presence of purine or pyrimidine analogues (9, 10) while the presence of specific combinations of nucleic acids and their precursors are necessary for the synthesis of particular enzymes in disrupted staphylococci (4). From these various lines of evidence it is clear that nucleic acids are concerned, not only in catalysis of protein synthesis in general, but also in organising that synthesis so that specific protein structures are formed. The problem now is to obtain experimental evidence which will enable us to elucidate the mechanism whereby nucleic acids are able to bring about organised protein synthesis.

When staphylococci are incubated with a $^{14}C$-labelled amino-acid and a source of energy, the amino-acid becomes incorporated into the protein of the cells. The properties of the reaction concerned in the incorporation of radio-glutamic acid have been studied in detail (11, 12); in this case, incorporation of radioactivity occurs only into glutamyl residues of the protein. The staphylococcus is an organism which has lost the ability to synthesise a wide variety of amino-acids and, consequently, can neither grow nor synthesise protein unless these amino-acids are supplied in the incubation medium. The incorporation which occurs when a single amino-acid is present in the incubation medium takes place in the absence of any measurable net synthesis of protein, and reasons have been put forward elsewhere (12) which indicate that the incorporation does not take place as a result of protein synthesis as such. The available evidence is compatible with the suggestion that incorporation can take place as the result of an exchange reaction occurring between the added amino-acid (or a metabolite thereof) and corresponding residues in the protein of the preparation. Incorporation of a labelled amino-acid cannot therefore be used as a measure of protein synthesis but studies of the former may throw light on the mechanism of the latter since it seems possible that protein formation may be superimposed on the exchange reactions when all the necessary amino-acids are available (12).

Incorporation will take place in cells which have been disrupted by exposure to supersonic vibration, and removal of nucleic acid from such preparations results in loss of their ability to incorporate certain amino-acids. This ability is restored by adding staphylococcal nucleic acid, either ribo- or deoxyribo-nucleic acid (RNA or DNA), to the incubation mixture. The restoration is species specific as nucleic acid preparations from yeast, liver, wheat germ, thymus or other bacteria prove to be inactive. Partial depletion of nucleic acid affects the incorporation of different amino-acids to differing extents : thus incorporation of glycine, aspartic acid or leucine is rapidly and markedly affected by partial removal of nucleic acid whereas the incorporation of alanine or lysine is affected, if at all, only by severe depletion of nucleic acid. It may be that each amino-acid responds to the presence of a specific portion of the nucleic acid structure and that some portions are more readily damaged or replaced than others. Incorporation reactions with different amino-acids are affected to widely differing degrees by the presence of inhibitors

such as chloramphenicol, penicillin or amino-acid analogues (12, 13, 14) so that the incorporation of each amino-acid must be considered as a separate and independent reaction.

In the case of the reactivation of incorporation by RNA it has been found that, for a variety of amino-acids, digestion of the nucleic acid with ribonuclease does not abolish the reactivation although the active material becomes dialysable. Further, digestion of RNA preparations from yeast, liver or *Clostridium welchii* renders the digests, as opposed to the whole nucleic acids, active in restoring glycine, leucine or aspartic acid incorporation in disrupted staphylococci. The activity of such digests might be due either to the presence within them of polynucleotide fragments mediating the incorporation, or to reconstitution of specific nucleic acid by the disrupted cells. To distinguish between these possibilities, digests of staphylococcal RNA have been fractionated by the chromatographic and ionophoretic procedures described by Markham and Smith (15)

The investigation of the fraction involved in the incorporation of aspartic acid serves to illustrate the procedure which has been developed. Staphylococci, suspended in buffered sucrose, are disrupted, fractionated and depleted of nucleic acid as previously described (11). The rate of $^{14}$C-labelled aspartic acid is then measured in the presence and absence of RNA and the maximum rate in the presence of RNA determined. A ribonuclease digest of staphylococcal RNA is run on paper in *iso*propanol-ammonia solvent, uridylic and guanylic acids being used as markers. The paper is then photographed in ultra-violet light at 260 mμ and the position of absorbing material marked on the paper. Usually six bands of material can be distinguished : band VI is marked by uridylic and band V by guanylic acid; bands I-IV run more slowly than guanylic acid. Material is eluted with water from each band separately and the optical density at 260 mμ determined; the ability of each eluate to replace RNA in the incorporation of aspartic acid is now determined. Fig. 1*a* shows that

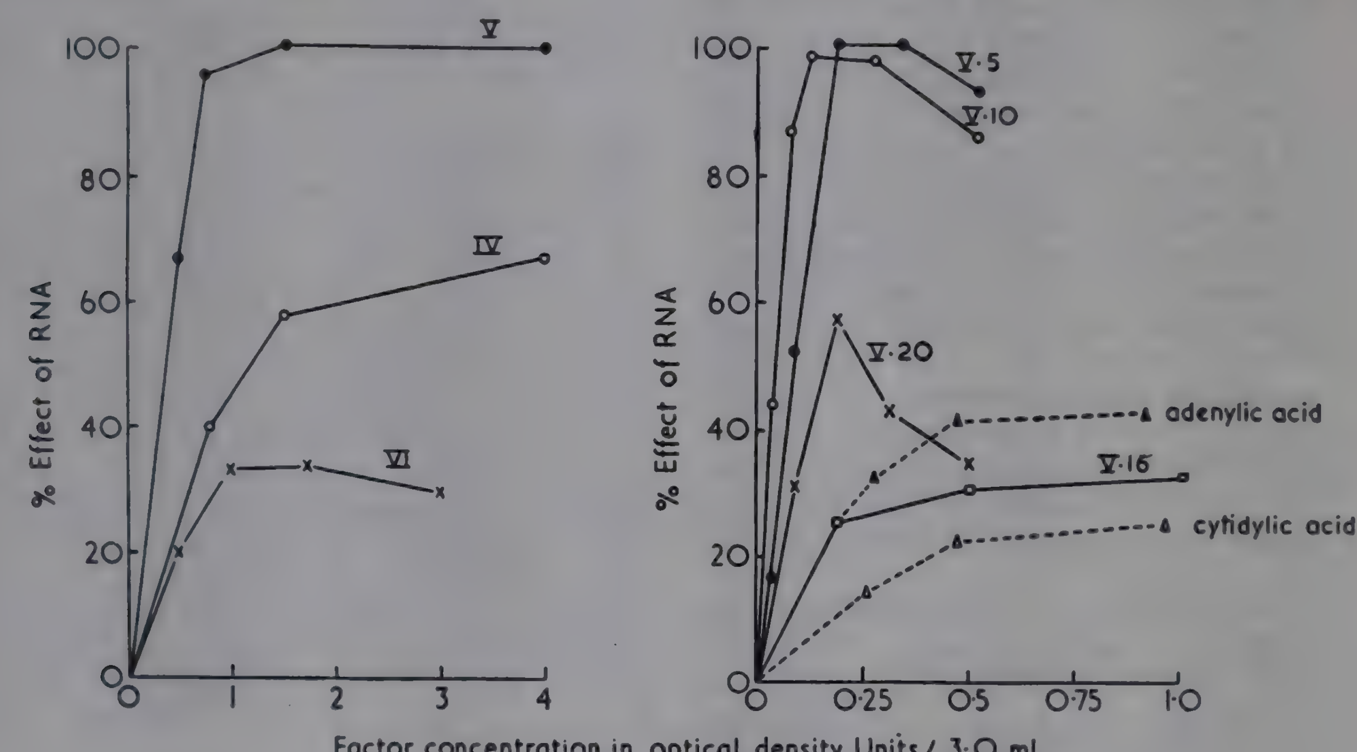


FIG. 1. — Incorporation of aspartic acid : replacement of RNA.

1*a*. Activity of materials separated from RNA digest by chromatography in *iso*propanol-ammonia (see text).

1*b*. Activity of components separated from chromatographic fraction V by ionophoresis at pH 3.5 and 20 volt/cm.; figures (V.5) represent distance moved from origin in 2 h.

and the ability of various fractions to replace RNA in the incorporation of specific amino-acids tested. Incorporation of a number of amino-acids has been tested and in several cases it has been possible to obtain single fractions from the digest which will promote the incorporation in place of RNA, the active fraction being different for each amino-acid. In all cases the separated fraction has been at least 100 times as effective as staphylococcal RNA when compared on an optical density basis. In 2 cases, the base composition of the polynucleotide fraction has been tentatively determined as shown below.

material from band V will completely replace RNA and, on an optical density basis, is *ca*. 20 times more effective than whole RNA. Material from either band VI or IV has some activity but, even at high concentrations, is never able to effect a complete replacement of RNA.

Each band contains a number of small polynucleotide fractions and these can be separated by ionophoresis. Markham and Smith (15) recommend ionophoresis at pH 3.5 and 20 volt/cm. If the material from band V is run on paper for 2 h. under these conditions, it separates into four or five components which can be located

by photography in the ultra-violet. The distance each component moves from the origin is constant under constant conditions (15) and can be used to characterise the component. Band V gives rise to two major components, moving at 10 and 16 cm. respectively, and three minor components moving at 5, 18 and 20 cm. although separation of the 18 cm. component is not always possible. Fig. 1*b* shows the action of these components in replacing RNA for aspartic acid incorporation and it can be seen that complete replacement is obtained with the 5 and 10 cm. substances. On a basis of optical density at 260 mμ the component V.10 (band V, ionophoretic movement 10 cm.) is the more active of the two substances which replace RNA. The material eluted from the 10 cm. position has been subjected to hydrolysis in N HCl for 1 h. at 100° C. and analysed for purine bases and pyrimidine nucleotides (Wyatt, 16); analysis shows the presence of adenine and cytidylic acid in equimolecular proportions. The general properties of the material thus agree with those given by Markham and Smith (15) effective for aspartic acid incorporation only; it has some activity in incorporation of glutamic acid, glycine or leucine but this activity gives at maximum 20-25 % replacement of RNA.

Fig. 2*a* and 2*b* show results obtained in a similar investigation carried out to isolate a nucleic acid factor which can replace RNA in the incorporation of glutamic acid. In this case RNA can be replaced by material running in band IV but not by substances in bands V or VI. On ionophoresis, band IV separates into a number of components all of which contain guanylic acid (G); only the fastest running component, substance IV.26, will completely replace RNA and, on an optical density basis, is 100-120 times as effective as RNA. The yield of this factor is small and identification is again only tentative; analysis after acid hydrolysis shows the presence of guanine and uridylic acid in proportions corresponding to a composition $GU_2$. This factor is without significant activity in promoting incorporation of aspartic acid.

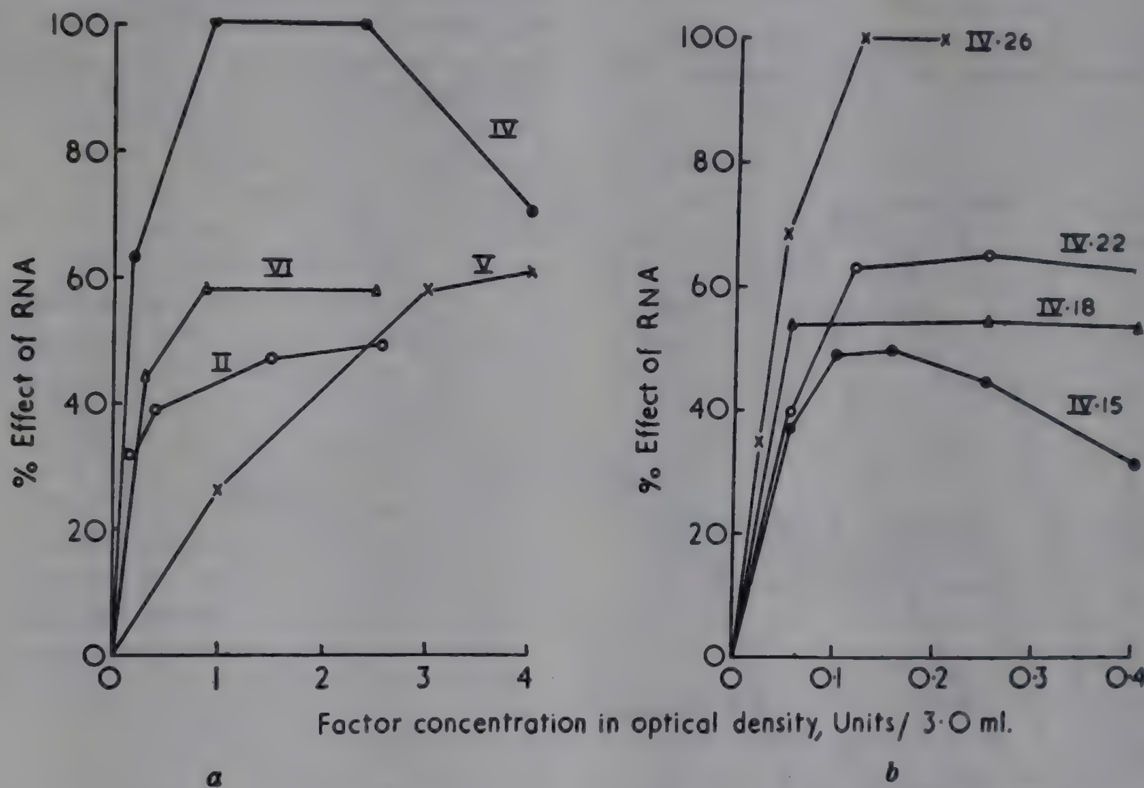


FIG. 2. — Incorporation of glutamic acid: replacement of RNA.

2*a*. Activity of materials separated from RNA digest by chromatography in *iso*propanol-ammonia.

2*b*. Activity of components separated from fraction IV by ionophoresis.

for adenine-cytosine-dinucleotide (AC) but until final identification has been made, it will be better to refer to the active material in terms of its characterisation as V.10. The component V.16, giving only partial replacement of RNA, agrees with the published data for AU (U = uridylic acid). The yield of component V.5 is small and accurate analysis has not yet been possible; it gives rise to adenine and cytidylic acid on hydrolysis and corresponds in properties to ACC. Adenylic acid and cytidylic acid have been prepared from yeast RNA and tested separately in the aspartic acid system; fig. 1*b* shows that neither can replace RNA although both have some activity, adenylic acid giving 40-45 % and cytidylic acid *ca.* 30 % replacement. The replacement of RNA by substance V.10, tentatively identified as AC, is

This type of analysis has been applied so far to the incorporation of 7 amino-acids and table I shows the positions of the various factors in the chromatographic bands and the ionophoretic mobility of the active materials at pH 3.5. In the case of phenylalanine it has not so far been possible to locate any single factor which will completely replace RNA; material in band I is fully effective but no single component separating on ionophoresis of that material has replaced RNA more than partially, although mixtures of such components are fully active. The factor active in replacing RNA for leucine incorporation can be characterised as V.18 and appears to run in front of the substance AU; insufficient material has as yet been obtained for any form of identification.

TABLE I.

*Properties of separated factors, obtained from ribonucleic acid digests, mediating the incorporation of specific amino-acids in disrupted staphylococci*

| Amino-acid incorporated | Active fractions of digest: Chromatographic band | Active fractions of digest: Ionophoretic movement at pH 3.5 and 20 V./cm. (cm.) | Concentration of factor giving half-saturation of incorporation rate (E units*/ml.) |
|---|---|---|---|
| Aspartic acid | V | 10 | 0.03 |
| Glutamic acid | IV | 25 | 0.02 |
| Leucine | V | 18 | 0.1 |
| Arginine | II | 0 | 0.01 |
| Glycine | VI | 2 | 0.006 |
| *Iso*leucine | VI | 2 | 0.015 |
| Phenylalanine | I | — | — |

(*) 1 E unit = amount of material having optical density at 260 mμ = 1. Half-saturation value for RNA = approx. 4 E units/ml.

was *iso*leucine and material which would completely replace RNA for its incorporation was located in band VI with ionophoretic mobility *ca.* 2 cm. — this corresponded with the material found active in the case of glycine incorporation. The relative activities towards glycine and *iso*leucine in a number of different preparations of VI.2 were determined and significant variations in the ratio of activities suggested that two different factors might be present in the VI.2 fraction. The fraction was concentrated and subjected to ionophoresis at pH 5 in acetate buffer, when it separated into two components, one strong one moving 15 cm. from the origin in 2 h. and a minor component remaining at the origin. All the activity towards incorporation of either glycine or *iso*leucine was found in the component remaining at the origin (VI.2.0). This component was eluted and run again in *iso*propanol-ammonia solvent when it separated into two substances, the faster of which (VI.2.0.B) ran in approximately the same position as cytidine and carried activity towards both glycine and *iso*leucine. Figure 3 shows the activity of this component, together with that of various nucleotides and nucleosides, towards glycine incorporation. The component VI.2.0.B has an absorption band in the ultra-violet with a maximum at

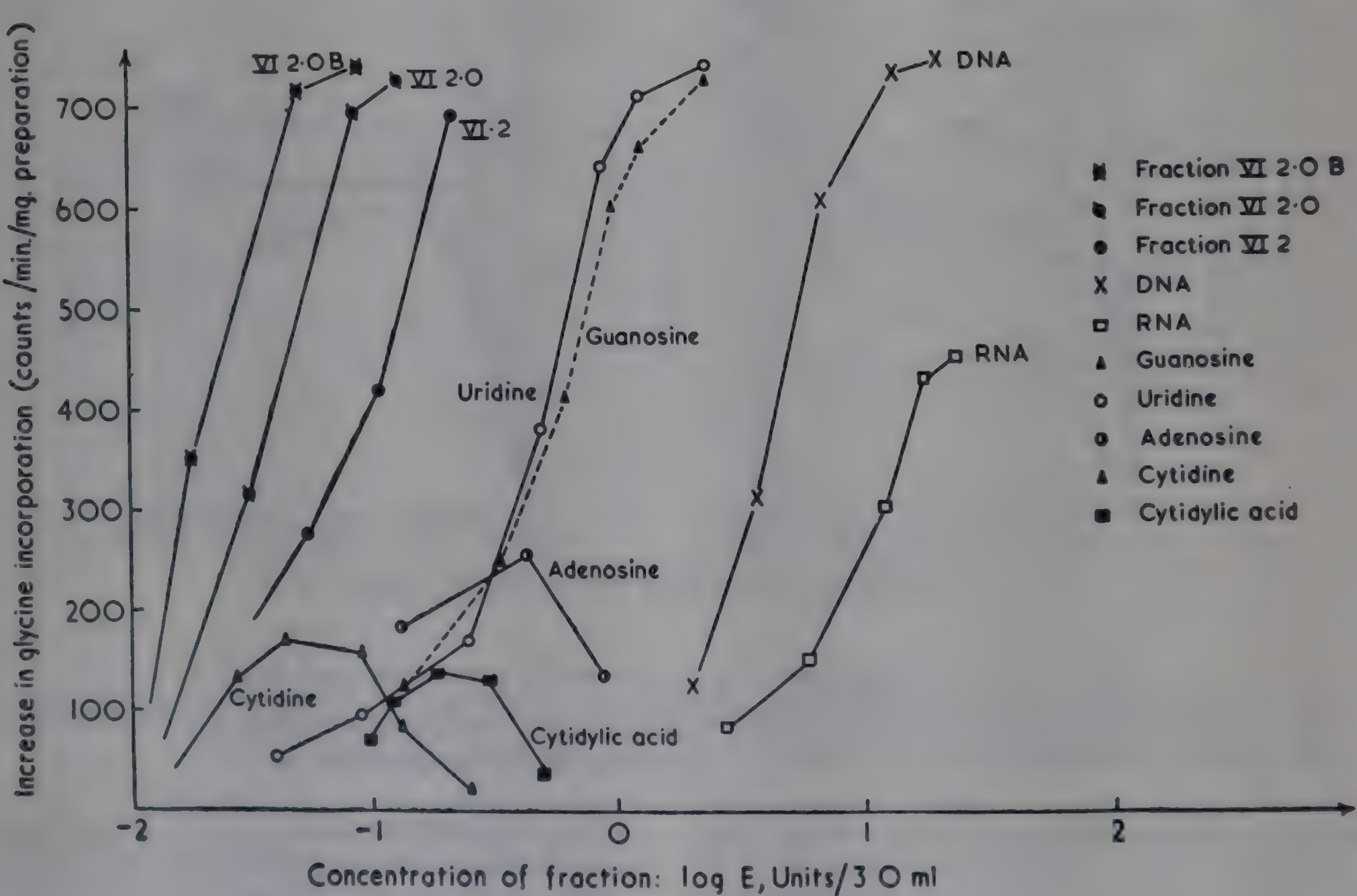


FIG. 3. — Incorporation of glycine : replacement of nucleic acid.
Effect of RNA, DNA and fractions separated from RNA digest on the incorporation of glycine (see text for explanation of fraction identifications).

This investigation was started with studies of the factors involved in the incorporation of glycine, aspartic acid, glutamic acid and leucine. Since active factors were isolated in each case from different fractions of the RNA digest, it seemed highly probable that a different factor would be found for each amino-acid studied. The next amino-acid whose incorporation was studied

270 mμ which suggests that it may contain cytidine but it cannot be replaced by cytidine or cytidylic acid or any of the known nucleosides or nucleotides so far tested. The component has no net electric charge at either pH 3.5 or 5.0 and it is improbable that it is any simple nucleotide or nucleoside. The yield of component VI.2.0.B is less than 0.1 % of the RNA used and

fig. 3 shows that, on an optical density basis, it is 5-700 times as effective as nucleic acid in promoting glycine incorporation. At the time of writing, insufficient of the factor has been prepared to allow further fractionation or attempts at analysis but it is hoped that further information will be available by the time of the Congress.

Fig. 3 shows that both uridine and guanosine have a marked effect in replacing RNA for glycine incorporation, although their efficiency is of a lower order than that of the factor prepared from the digest.

It has been noted previously that DNA is more effective than RNA in promoting amino-acid incorporation in nucleic acid-depleted preparations (12), both the rate and the final amount of incorporation attained being higher in the presence of DNA than RNA. This difference is shown again in fig. 3 and it is interesting to see that the glycine factor, isolated from RNA digests, promotes incorporation to that level attained in the presence of DNA. This would suggest that the activity of the factor is in some way restricted as long as it is part of the RNA complex.

Summarising the position at this stage of the investigation, it can be said that the whole nucleic acid complex is not necessary for the incorporation of any particular amino-acid, and that RNA can be replaced by small fragments obtained by ribonuclease digestion of the whole RNA structure. Some of these fragments appear to be small polynucleotides, although the factors so far isolated may be mixtures, and the nature of the highly active glycine factor has yet to be determined. It also seems probable that there is specificity between the fragments and the amino-acids whose incorporation they promote, but the degree of this specificity is uncertain while the glycine-*iso*leucine factor remains unresolved. Proponents of the template theory of nucleic acid function (17, 18) suggest that specific groupings of nucleotides act as combining points for specific amino-acids, so that the sequence of bases along the polynucleotide chain determines the sequence of amino-acids in the protein which will be formed on that chain as template. Such a mechanism might underlie the experimental findings described here, the factors isolated from ribonuclease digests representing specific groupings forming loci for the combination of specific amino-acids, and bringing about exchange of those amino-acids with corresponding residues in proteins of the preparation.

Whether deductions concerning protein synthesis, which apparently requires the intact RNA structure (4), can be made from these findings depends upon the relationship between protein synthesis and incorporation reactions. If the template theory has a basis in fact, then there must be a stage in protein synthesis when amino-acid residues are combined with their specific loci on the template and not with each other through peptide bonds. Exchange reactions between amino-acids in the medium and corresponding residues on the template could then occur and lead to incorporation of labelled amino-acids in the absence of net synthesis. Incorporation by exchange may thus form a part of the protein synthesising mechanism; complete nucleic acid may present a linked series of specific loci each of which corresponds to the position of a specific amino-acid in a protein sequence. The promotion of incorporation by exchange would thus depend upon the order of the loci in a particular nucleic acid, and the species specificity of undigested nucleic acids in this respect be explained.

The disrupted staphylococcal cell provides a system in which these ideas can be experimentally tested.

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## Some biochemical aspects of the cell nucleus


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(*Received 3 August* 1955)


Before coming to the biochemistry of the cell nucleus we should consider briefly some of the conclusions reached by biologists in their investigations of the cell nucleus. In the chromosomes of a higher organism a certain genetic continuity has long been recognized by biologists. Genetic continuity is made possible by the precision with which the germinal material in chromosomes is divided at mitosis. An example of genetic

continuity is the fact that the details of microscopic structure visible in the chromosomes of the salivary gland or the Malpighian tube of a Drosophila larva correspond to the genetic constitution of the chromosomes of the egg and sperm which combined to form this larva. Here is presented a striking contrast between the genetic continuity observed in the chromosomes and the morphological and physiological diversity of the cells in which these chromosomes are embedded. The chemistry of chromosomes must be considered in relation both to genetic continuity and cellular differentiation.

At the present time investigation of the chemistry of chromosomes is being carried out with the most varied materials : the phage, bacteria, amoeba and cells of higher animals, to mention only some. It can already be seen that investigation of each type of organism is making its special contribution to the general fund of knowledge of chromosomes. Each organism offers special attractions to the investigator. Since this report deals with work done on cells of higher animals some of the attractions they present will be mentioned : in these cells genetic continuity and its contrast with differentiation are so clearly perceived that they are constantly on the mind of the investigator; and in these cells the distinction between nucleus and cytoplasm is obvious, so that it is possible to make preparations of isolated nuclei.

It is now well known from analyses of cell nuclei, as well as from investigations on the phage and on the transformation of pneumococci, that DNA is the chromosomal constituent responsible for genetic continuity. It has been found that the quantity of DNA per set of chromosomes is a constant in the different cell types of a higher organism and that the constant is a characteristic of the species. An example is given in table I

TABLE I

*DNA content of various nuclei of the fowl (expressed as mg. × $10^{-9}$/nucleus)*

| Determinations by | Erythrocyte | Liver | Kidney | Spleen | Heart | Pancreas | Sperm |
|---|---|---|---|---|---|---|---|
| Mirsky and Ris, 1949 | 2.34 | 2.39 | — | — | — | — | 1.26 |
| Davidson, Leslie, Smellie and Thomson, 1950 . . . | 2.49 | 2.56 | 2.20 | 2.54 | 2.45 | 2.61 | — |

where it is seen that in the domestic fowl diploid cells as different from each other as are erythrocytes and hepatic cells a constant quantity of DNA per nucleus is present; and this is just twice the quantity present in the haploid sperm cell. The constancy of DNA per set of chromosomes for such different cells of the fowl corresponds to the genetic continuity that has been observed in chromosomes of different cells of Drosophila.

The analyses that have just been mentioned were done by conventional biochemical procedures on suspensions containing known numbers of cells or isolated nuclei. A comparison of such analyses with those done on single nuclei in which chromosomes are stained by the Feulgen procedure has rigorously demonstrated that under certain conditions the microspectrophotometric determination of DNA gives reliable results (1). Such quantitative Feulgen determinations have greatly extended the scope of DNA analyses. In this way it has been found that in higher degrees of ploidy, as well as in haploid and diploid nuclei, the quantity of DNA is a constant for each chromosome set of a given organism, table II (1, 2). Feulgen determinations have also shown that in the nuclei of the ovum and sperm of Ascaris equal quantities of DNA are present (3).

TABLE II

*Size of nuclei (polyploidy) and intensity of Feulgen reaction in rat liver*

| Size of nuclei | $E$ × area (n = 10) | Ratio |
|---|---|---|
| Smallest nuclei . . . . . . . . . . . | 5.5 ± 0.1 | 1 |
| Medium sized nuclei . . . . . . . . | 10.4 ± 0.1 | 1.9 |
| Largest nuclei . . . . . . . . . . . | 19.9 ± 0.2 | 3.6 |

Fixation : 10 % formalin.

Recently improvements by Davies, Walker and Wilkins in the technique of microspectrophotometry have greatly increased the accuracy of DNA determinations on Feulgen preparations. There have also recently been reported several apparent exceptions to DNA constancy (4, 5).

Histones are basic proteins combined with DNA in nuclei of many plant and animal cells. A comparison of their distribution with that of DNA in the various nuclei of an organism is of much interest at present partly because of some recent advances in knowledge of histones. Within the past year reports from three laboratories have shown that in the histones extracted from nuclei of a number of tissues there are in addition to histones of high arginine content also histones containing as much as 35 % lysine and only 3.9 % arginine (6, 7, 8, 9). In the experiments of Crampton, Moore and Stein, fractionation was carried out chromatographically using a column of IRC-50. When a crude histone preparation of calf thymus was put on the column much of the histone was not subsequently eluted. In the eluate (containing about one-third of the histone material placed on the column) there were two main peaks, one of which corresponded to a histone of high lysine content, the other to an arginine-rich histone. When a crude preparation of beef liver histone was placed on the column precisely the same results were obtained, with the two main peaks of the eluate coming at just the same positions. Furthermore amino acid analyses on each of the two histones of the beef thymus eluate and on the two homologous histones of beef liver gave practically identical results. A far more searching test

of just how closely the corresponding histones of thymus and liver resemble each other is to digest them with trypsin and then carry out a chromatographic analysis of the large number of distinctly different basic peptides produced from each histone. The results of such peptide analyses by Crampton, Moore and Stein showed that the corresponding histones of beef and thymus are at present indistinguishable from each other.

There is a certain resemblance between the constancy found in the DNA content per set of chromosomes in nuclei of different cell types of an organism and the constancy in composition of the various histones combined with DNA in those nuclei. And yet there is a distinction to be noted in the distribution of DNA and histones. It has been known since the time of Miescher that in the sperm of certain fishes the basic proteins combined with DNA are protamines. Only a little later histones were discovered in goose erythrocytes by Kossel. Let us indicate these facts in table III. Some years ago the

TABLE III

| | Erythrocyte nucleus | Sperm nucleus |
|---|---|---|
| Salmon . . . . | histone (?) | protamine |
| Fowl . . . . . | histone | protamine (?) |

spaces in the table in which question marks are placed were filled (10). We found that salmon erythrocyte nuclei contain histone and no protamine and that fowl sperm nuclei contain a protamine-like protein, which we called gallin. There is no histone in the sperm nuclei of either the salmon or the fowl, although histones are present in their somatic nuclei. Is it possible that some essential configurations of histones are also present in protamines ? In any case it seems likely that some chromosomal functions which require the presence of histones are not performed in sperm nuclei : and in this connection it should be recalled that there are genetic experiments indicating a certain absence of gene action in sperm cells, although of course, they do transmit genes.

In addition to histones there are other proteins associated with DNA in chromosomes. These non-histone proteins will be referred to later in the present report.

DNA and histone are nuclear components which, despite striking signs of cell differentiation, exhibit a certain constancy in the different cells of an organism. There is of course abundant evidence that there are other nuclear components which vary. Nuclei of different cell types are usually readily distinguished from each other microscopically; and there are also many observations by cytologists of changes in appearance of nuclei during such physiological processes as glandular secretion.

Investigation of the enzymes present in nuclei have provided many examples of variations in the chemical composition of different nuclei of the same organism (11). For a study of the water-soluble enzymes of the cell nucleus the most reliable procedure at present is to work with enzymes of nuclei isolated in non aqueous media. Nuclei which appear to be clean when examined microscopically have been isolated in non-aqueous media from liver, thymus, kidney, pancreas, heart and other tissues. The absence from the isolated nuclei of certain components present in the cells from which they have been derived is itself an excellent indication that the nuclei are not grossly contaminated by cytoplasmic constituents. In this way, for example, the absence of lipase, amylase and trypsinogen from isolated pancreas nuclei or of myoglobin from isolated cardiac nuclei serve as useful signs that the nuclei are clean. Investigation of the enzymes of nuclei demonstrate decisively that the nucleus is a differentiated structure which varies from tissue to tissue. This conclusion is in line with results of the remarkable experiments recently described by Briggs and King in which somatic nuclei are used to fertilize the enucleated frog egg (12). Observations on nuclear differentiation emphasize, by contrast, the constancy in nuclei of the same organism of DNA and to a more limited extent, of histone.

What is the role of DNA in the biochemical functions of the nucleus ? Recent experiments with nuclei isolated from calf thymus are steps towards an answer to this question. The nuclei were isolated in sucrose solutions. When examined with the ordinary light microscope, with the phase-contrast microscope, with the interference microscope and with the electron microscope the thymus nuclei used in these experiments were found to be free of adhering cytoplasm. Although nuclei isolated in this way from liver are ordinarily grossly contaminated with cell debris and have had much of their water-soluble contents washed away, thymus nuclei are remarkably clean and have lost surprisingly little of their water-soluble material. The extent to which water-soluble constituents are washed out of nuclei in the course of isolation in sucrose solutions can be determined by comparing these nuclei with those isolated in non-aqueous media, into which the water-soluble constituents do not pass. In this way it has been shown that certain water-soluble enzymes which are extracted from liver nuclei by sucrose solutions are not extracted from thymus nuclei. Furthermore, the total protein content of thymus nuclei, as determined by the DNA-protein ratio, is the same whether they are isolated in non-aqueous media or in a sucrose solution (13).

The extent to which water-soluble constituents are retained by thymus nuclei in sucrose solutions is indeed surprising, for in recent experiments we find that the bulk of the mononucleotides originally present in the nuclei is retained (14). Chromatographic analysis on columns of Dowex-1 showed that the mononucleotides present in thymus nuclei isolated in non-aqueous media are both qualitatively and quantitatively about the same as those present in the cytoplasm. In both nucleus and cytoplasm the mononucleotides found are cytosine, uracil, guanine and adenine compounds, with the latter predominating. About 60 % of these nucleotides are retained after isolation of nuclei in a sucrose solution. Phosphorylation of the nucleotides to their triphosphate forms occurs rapidly in thymus nuclei isolated in sucrose, and this seems to be an oxidative process.

Even before the analyses on isolated nuclei that have just been mentioned were made, nucleotides soluble in

fixatives had been detected by Davies (15), Walker and Yates (16) in the course of their microspectrophotometric observations on nuclei in tissue cultures. The quantity of nucleotides found by them was approximately the same as what we found in thymus nuclei isolated in non-aqueous media. The English investigators supposed that these 'lower' nucleotides are precursors of DNA. Now that their chemical identity has been recognized it is evident that, as with cytoplasmic mononucleotides, they play a part as triphosphates in numerous processes. It is likely that the nuclear mononucleotides play a part in the synthesis of protein that occurs in the nucleus, a process which will now be described.

Experiments reported a year ago by my associate Dr. Allfrey showed that thymus nuclei isolated in sucrose are able rapidly to incorporate amino acids labelled with $^{14}C$ into their proteins (17). These experiments have since been continued (18). An important condition for rapid incorporation is the presence of sodium ions and, as shown in figure 1, uptake of amino acid in relation

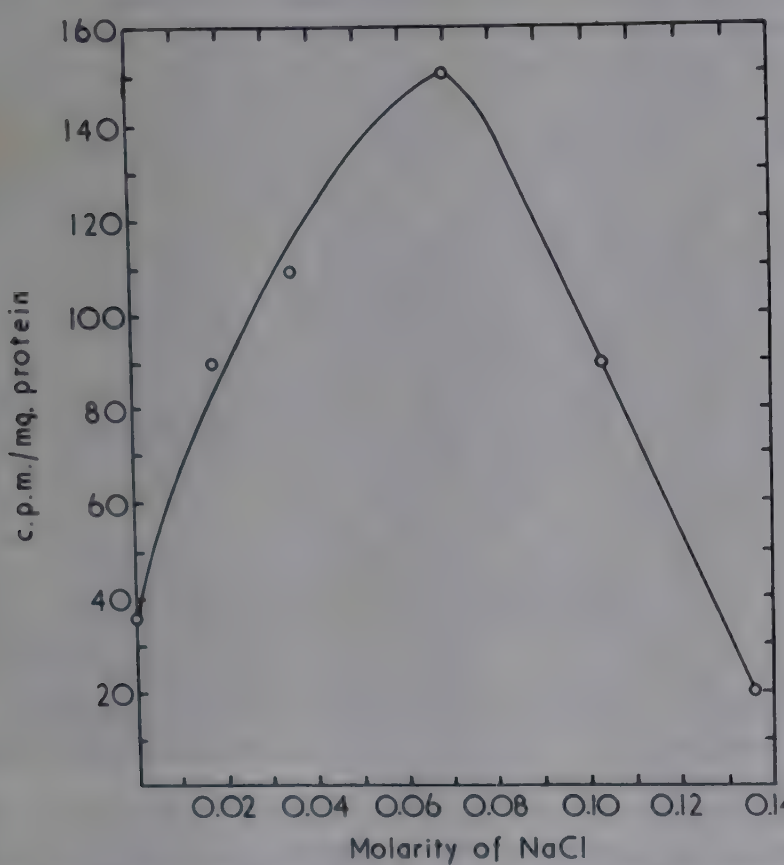


**Fig. 1. — Glycine-1-$^{14}C$ incorporation *vs.* NaCl concentration; $K^+$ concentration = 0.0135 M.**

to sodium concentration shows a well-defined maximum. It can be seen in figure 2 that potassium inhibits. In every amino acid uptake experiment with isolated thymus nuclei the effects of sodium and potassium must be considered. It is also necessary to recognize the lability of the nuclei, for activity varies from one preparation to the next and declines as a preparation is kept, even in the cold. Activity is destroyed by freezing, heating to 90°C. for 3 minutes, or raising the sucrose concentration much above 0.25 M.

Uptake requires oxygen; it does not occur in nitrogen and is inhibited by cyanide. We do not yet know what substance combines with oxygen to yield the energy required for uptake. Addition of glucose increases uptake by about 20 %. The main source of energy is some material originally present in the nuclei. It should be noted that uptake is not inhibited by chloramphenicol but is inhibited by cortisone.

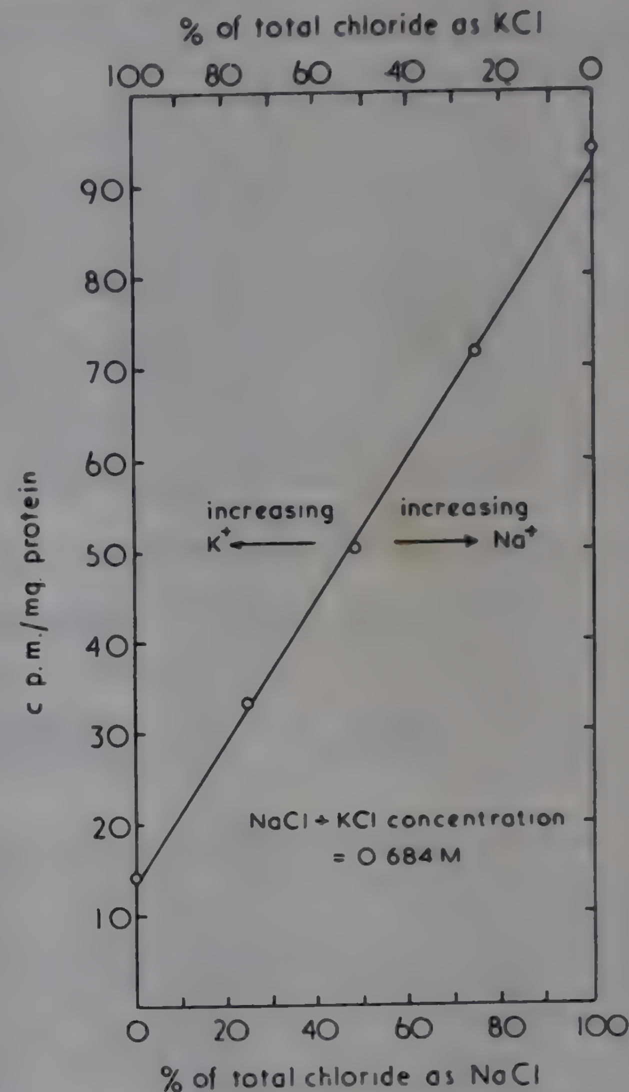


**Fig. 2. — Glycine-1-$^{14}C$ incorporation *vs.* NaCl : KCl ratio; NaCl + KCl concentration = 0,684 M.**

Both labelled alanine and glycine, the only amino acids that have been used so far, are incorporated. The uptake of L-alanine is not affected by the presence of D-alanine, even in high concentration. The uptake of labelled L-alanine is, of course, diminished by the presence of unlabelled L-alanine, but once some labelled L-alanine has been incorporated, it is not released by addition of unlabelled L-alanine. These observations show that uptake is not reversible, and that it is therefore not merely an 'exchange reaction'. Uptake of amino acid may be considered to be essentially 'protein synthesis'.

Synthetic activity of nuclei is dependent upon the presence in them of DNA, for if the DNA is broken down by action of DNAase there is a sharp drop in the rate of amino acid uptake. These experiments show that the biochemical function of DNA, the chromosomal material associated with genetic continuity, is concerned with protein synthesis. There is evidence that the decrease in activity due to the effect of DNAase can be restored to some extent by addition of thymus DNA. Activity is not impaired by addition to the nuclei of methyl green or histone. Since both of these substances combine with some readily available phosphoric acid groups of

DNA, it may be said that blocking of these groups does not stop the uptake of amino acids.

Into which of the numerous protein fractions of the thymus nucleus does amino acid incorporation take place ? There is first of all the protein fraction soluble in 0.1 M salt at neutrality (I). This accounts for somewhat less than 10 % of the mass of the thymus nucleus. Most of the material in the nucleus can then be dissolved in M NaCl, leaving a residue of protein that amounts again to somewhat less than 10 % of the original nuclear mass (II). The protein dissolved along with DNA in M NaCl consists of histones and non-histone proteins. We have fractionated this mixture with saturated NaCl and have obtained two fractions of non-histone protein, one of which is rather gelatinous and is separated from DNA by concentrated NaCl (III); the other fraction remains attached to DNA under these conditions (IV). Fractions II, III and IV correspond to the non-histone fraction of the chromosomes which in our previous work we have referred to as 'residual protein', the protein remaining in the chromosome after removal of DNA and histones. It need hardly be said that we are only at the beginning of the fractionation of the proteins of the thymus nucleus.

Incorporation of labelled amino acid into a mixed histone fraction is far lower than into any other protein fraction of the nucleus. Incorporation into fraction IV, the protein which was not removed from DNA by saturated NaCl, was the highest of any fraction, being nearly 7 times that into histone. Incorporation into fraction I was also very high. This protein is associated with about 1/3 of the RNA of the nucleus and may well be diffusible. It is tempting to suppose that fraction I represents material, now on its way out into the cytoplasm, that was synthesized in association with DNA. It should be noted that another large part of the nuclear RNA is associated with fraction II.

If we consider what we now about protein synthesis in isolated nuclei along with what is already known about protein synthesis in other isolated cell components (19, 20, 21) it is clear that in the cell synthesis of protein may occur both in the nucleus and in the cytoplasm. In the isolated cytoplasmic system the use of RNAase has shown that protein synthesis depends upon the presence of RNA (21, 22) just as in isolated nuclei the use of DNAase has shown that protein synthesis depends upon the presence of DNA. Isolated nuclei may also provide us with an opportunity to study the role of nuclear RNA in nuclear activity.

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## A note on the incorporation of precursors into cell constituents

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(*Received* 9 *September* 1955)

Dr. Gale and Dr. Mirsky are to be congratulated on the very beautiful work which they have described.

Dr. Gale has evolved a very remarkable system in his disrupted staphylococcal preparations which have proved capable of retaining a quite surprising amount of biological activity after drastic treatment, both chemical and biological. The fact that such preparations in which the integrity of the bacterial cell has been lost and in which the conditions are far from what one would normally regard as physiological, are still capable not

merely of incorporating amino acids but of actually synthesising protein is a very striking and quite unexpected observation. It suggests that the protein synthesising mechanisms of the cell are perhaps not so delicate as has hitherto been supposed, at least in bacterial cells. In animal cells, it has certainly been generally considered that the protein synthesising mechanism is delicate enough to be easily upset by relatively minor alterations in physiological state.

But the real significance of Dr. Gale's recent work is that he has for the first time produced definite evidence that the incorporation of individual amino acids can be controlled by clearly defined combinations of nucleotides. One may therefore conclude that the sequence of nucleotides found in any particular ribonucleic acid will regulate the incorporation of a group of amino acids with the requisite pattern to form a specific protein. This is a very important advance indeed for it gives us greater insight than has hitherto been possible into the mechanism by which nucleic acids may act in protein synthesis. Indeed it gives an experimental basis to some of the theoretical views of Gamow (1) and others on the question of 'coding'.

What one would now like to know is the relative roles of RNA and DNA for Gale and Folkes (2) have shown that DNA as well as RNA can restore activity to staphylococcal preparations depleted of nucleic acid. Does DNA really act as a template for the synthesis of RNA ? Or does it in some way control the activity of RNA and especially the remarkably high metabolic activity of nuclear RNA ?

Dr. Mirsky has drawn attention to the proteins associated with both RNA and DNA and particularly to the proteins of the cell nucleus. These observations show an interesting correlation with some of our own results obtained recently in Glasgow in which we have found that the rate of incorporation of $^{14}$C-formate into the non-histone protein of the nucleus relative to the rate of incorporation into the histone is greatly increased in cells which are undergoing rapid cell division.

Both Dr. Gale and Dr. Mirsky have used incorporation procedures and in this connection I should like to make two comments based on our own observations.

The first is that the pattern of incorporation into the same cell constituent may vary greatly with the physiological conditions and with the type of cell employed. For example rabbit bone marrow readily incorporated $^{14}$C-formate into DNA *in vivo* both into the purines and into the methyl group of thymine. When rabbit bone marrow is removed from the animal and suspended in a suitable saline buffer it will still incorporate $^{14}$C-formate almost equally rapidly into the thymine but the incorporation into the purines is drastically reduced. This observation was first made by Totter and colleagues (3, 4) and has been confirmed by ourselves (table I). In this particular case the conditions *in vitro* (mechanical dispersion of the cells in a salt mixture) are far from physiological and the preparation is in effect slowly dying. Hence impairment of incorporation is not surprising. What is surprising is that incorporation into the thymine is so good.

But we have been able to make similar observations with cells of the Ehrlich ascites carcinoma (table I). *In vivo*, these cells incorporate $^{14}$C-formate readily into the DNA purines and into the thymine. When the suspension of cells in ascitic fluid is drawn off and incubated *in vitro* immediately without any additions except the formate, *i. e.* when the *in vitro* conditions are as

TABLE I

*Incorporation of $^{14}$C-formate during 2 hours (c.p.m./µ-mole)*

| | DNA | nRNA | cRNA |
|---|---|---|---|
| Rabbit bone marrow | | | |
| *In vivo* : adenine | 5842 | 7164 | 3605 |
| guanine | 3736 | 4276 | 1721 |
| thymine | 6711 | — | — |
| *In vitro* : adenine | 123 | 534 | — |
| guanine | 145 | 429 | — |
| thymine | 4699 | — | — |
| Ehrlich ascites mouse tumour | | | |
| *In vivo* : adenine | 1460 | 9780 | 2200 |
| guanine | 3980 | 11800 | 1400 |
| thymine | 3250 | — | — |
| *In vitro* : adenine | 68 | 1500 | 113 |
| guanine | 330 | 2460 | 160 |
| thymine | 2960 | — | — |

DNA = desoxyribosenucleic acid ; nRNA = nuclear ribosenucleic acid ; cRNA = cytoplasmic ribosenucleic acid.

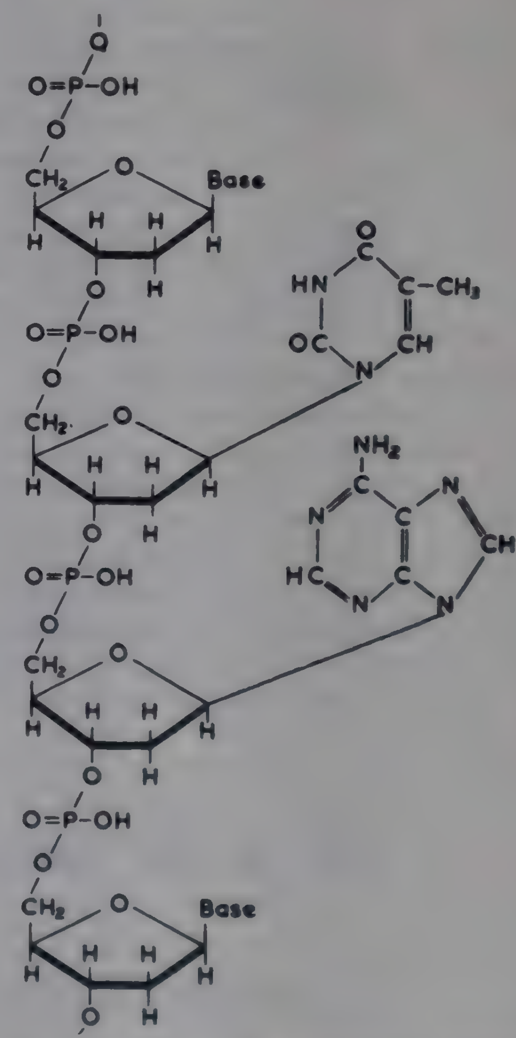

FIG. 1. — Portion of the DNA molecule showing nucleotide residues containing thymine and adenine.

nearly as possible equivalent to those *in vivo*, incorporation into thymine is almost as effective as before but incorporation into the purines in both DNA and RNA is greatly reduced.

Moreover the pattern of incorporation *in vivo* differs between bone marrow and ascites cells. Both are rapidly proliferating tissues and both show high incorporation of $^{14}$C-formate into DNA as well as into RNA. But in the ascites cells incorporation into guanine is greater than into adenine whereas in bone marrow the reverse is the case. In ascites cells also the activities of the purine bases in the nuclear RNA relative to the activity in the DNA is much greater than in bone marrow.

Patterns of incorporation may vary therefore with the conditions and with the type of cell.

Indeed the pattern on incorporation of different precursors into the DNA of the ascites cells *in vitro* is rather surprising. Incorporation of $^{32}$P is very good indeed; that of $^{14}$C-formate is high into thymine and negligible into the DNA purines. $^{14}$C-glycine is very poorly incorporated into the DNA purines while on the other hand $^{14}$C-adenine is taken up very rapidly (fig. 1). Thus labelled phosphate and adenine are well taken up whereas glycine and formate are poorly incorporated into the purines. This is the reverse of what might have been expected on the basis of earlier experiments on resting tissues.

The second point relates to the influence of the cytoplasm on incorporation into the nucleus. This is well illustrated in some as yet incomplete experiments carried out in our laboratory by Mr. R. Logan using composite homogenates made up of isolated nuclei and isolated cytoplasmic fractions from rabbit liver tissue put together in the presence of ATP, cytochrome *c*, DPN, $Mg^{++}$ ions, sodium malate and glycine buffer. In the presence of radioactive inorganic phosphate but without other cytoplasmic material incorporation into nuclear RNA or DNA is negligible. Addition of non-radioactive cell sap promotes incorporation into both RNA and DNA, particularly the latter. Replacement of cell sap by particulate fractions is ineffective.

When the nuclei are incubated with the acid soluble fraction obtained from the cell sap from animals which have received $^{32}$P, incorporation is negligible, as with radioactive inorganic phosphate. But when the acid soluble fraction is supplemented by non-radioactive cell sap incorporation into nuclear RNA is enhanced slightly while incorporation into DNA is enormously increased. It is clear therefore (*i*) that cell sap contains a non-dialysable factor which promotes incorporation of $^{32}$P into DNA and (*ii*) that the acid soluble fraction contains a more effective precursor than inorganic phosphate.

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# Das kontraktile System von Muskel und Zellen

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(*Eingegangen am* 20 *April* 1955)

## I

Die chemische Analyse des lebenden Muskels (Meyerhof 1; Lohmann, 2; Embden, 3; Parnas, 4; P. und G. P. Eggleton, 5; Fiske und Subbarow, 6) brachte uns die Kenntnis aller wesentlichen chemischen Reaktionen, die im Betriebsstoffwechsel des Muskels eine Rolle spielen.

Die diesen chemischen Versuchen übergeordnete thermodynamische Analyse von A. V. Hill und O. Meyerhof gab uns gleichzeitig die energetische Bilanz dieser Reaktionen des Betriebsstoffwechsels im Rahmen der Aktivität des lebenden Muskels.

Die energetische Bilanz ermöglichte ausserdem die Zuordnung der einzelnen Prozesse zu bestimmten Phasen der Muskeltätigkeit, d. h. zur Arbeitsphase und zur Erholungsphase.

Die hierdurch gegebene Reihenfolge der einzelnen Reaktionen wurde durch gewisse physikalische Untersuchungen über die Aenderungen der optischen Transparenz des tätigen Muskels (von Muralt, 7), der Elektrostriktion (Meyerhof und Möhle, 8) und besonders auch des pH (Dubuisson, 9) verfeinert.

Andere physikalische Untersuchungen am lebenden Muskel (A. V. Hill *et al.*, 10) zeigten die Existenz der sogenannten « Extraenergie ». Die Abgabe dieser « Extraenergie » ist wichtig, weil auch sie von einer erschöpfenden Theorie der Muskelkontraktion erklärt werden muss, während sie von keiner chemisch begründeten Kontraktionstheorie auch nur berücksichtig wird.

Die Erkenntnisse, die hier skizziert wurden, liegen praktisch vollständig seit etwa 15 Jahren vor. Sie sind bis 1954 durch Untersuchungen des lebenden Muskels nicht mehr wesentlich vermehrt worden. Diese Erkenntnisse laufen darauf hinaus, dass die ATP-Hydrolyse die erste bekannte exergonische Reaktion des Arbeitsstoffwechsels ist; diese Erkenntnisse lassen es aber offen, ob die Energie der ATP-Spaltung unmittelbar in mechanische Arbeit verwandelt wird oder ob auch sie nur der Reversion eines noch unbekannten Vorganges dient, der seinerseits erst die mechanische Arbeit liefert.

Denn der sehr kurze Zeitraum, in dem im lebenden Muskel mechanische Arbeit erzeugt wird, kann mit Hilfe chemischer Analyse nicht sicher von dem vorangehenden Augenblick der Erregung und dem nachfolgenden Zeitraum der Restitution unterschieden werden.

## II

Infolgedessen verdanken wir die nächste Gruppe von Erkenntnissen der Anwendung einer neuen und ganz andersartigen experimentellen Technik : etwa seit 15 Jahren wird vorzugsweise das Verhalten des isolierten Kontraktionsvorganges untersucht.

Die experimentelle Isolierung des Kontraktionsvorganges geht aus von den Entdeckungen von Engelhardt (11), von Needham (12) und vor allem von Szent-Györgyi (12*a*). Denn diese Autoren fanden, dass die nach Edsall (13) und Weber (14) isolierten und gereinigten kontraktilen Proteine ATP spalten und gleichzeitig ihren eigenen kolloiden Zustand ändern. Diese kolloidalen Zustandsänderungen sind im Gel- und im Solzustand verschieden. Die Anderungen des Solzustandes auf ATP-Zusatz — z. B. Abnahme der Viskosität, des light-scattering, der Doppelbrechung — waren sehr lange in ihrer Bedeutung umstritten. Dagegen hat sich auf den Spuren der erwähnten Pioniere in der Zwischenzeit einwandfrei zeigen lassen, dass die ATP-induzierten Anderungen des Gelzustandes auf eine arbeitsleistende molekulare Kontraktion hinauslaufen (15).

Werden die kontraktilen Proteine nämlich zu Fäden mit wohlorientierter molekularer Feinstruktur versponnen, so verkürzen sie sich auf Zusatz von ATP und Mg unter Spannung und leisten Arbeit. Solche arbeitsleistenden Fäden sind bisher hergestellt aus dem gereinigten kontraktilen Protein — dem Aktomyosin — des Säugermuskels (16, 17, 18) und aus dem gereinigten kontraktilen Protein glatter Molluskenmuskeln (19). Wir nennen solche Fäden weiterhin Fadenmodelle.

Die Kontraktion solcher Fäden gleicht qualitativ völlig und in einigen Zügen auch quantitativ der Kontraktion der Muskeln, aus denen das kontraktile Protein, jeweils gewonnen ist. Dagegen ist die Arbeitsleistung infolge geringerer maximaler Spannung und geringerer Verkürzungsgeschwindigkeit wesentlich kleiner als die Arbeitsleistung des betreffenden lebenden Muskels (17, 19). Die Kontraktion der Fäden stellt demnach zwar einen isolierten, aber gleichzeitig quantitativ modifizierten Kontraktionszyklus dar.

Solche Modifikation des vitalen Kontraktionszyklus verschwinden weitgehend oder ganz, wenn die Isolierung des Kontraktionsvorganges durch Extraktion des Gewebes

mit wässerigen Lösungen oder Glyzerin-Wasser-Mischungen stattfindet. Dieses Verfahren ist von Varga (20) und A. Szent-Györgyi (21) für Muskelfasern und von meinem Mitarbeiter Hoffmann-Berling (22) für einzelne Zellen eingeführt worden. Wir bezeichnen solche zu Erregung und Erholung (Restitution) unfähige Strukturen aus kontraktilem Protein und Stützgewebe als Fasermodelle und Zellmodelle.

Die Kontraktion der Zell- und Fasermodelle gleicht der Kontraktion des lebenden Gebildes, aus dem die Modelle hergestellt sind, so weitgehend, dass die beiden Kontraktionsvorgänge vielfach nicht unterscheidbar sind. Expandierte mesenchymale und ektodermale Zellen und Gewebekulturen kontrahieren sich vor dem nächsten Teilungsschritt mit genau der gleichen Geschwindigkeit

ABB. 1. — Kontraktion von Zellmodellen (Amnionfibroblast) durch $2 \times 10^{-3}$ M ATP bei pH 7.0 und $I = 0.14$ Γ, 37° C. (nach Hoffmann Berling, 23) 28 Tage extrahierte Modelle; links : ohne ATP; rechts : 12 Min. nach Zugabe von ATP.

und in genau dem gleichen Umfang im lebenden Zustand wie im Modellzustand (Abb. 1). Ebenso stimmt die Geschwindigkeit und der Umfang der äquatorialen Kontraktion während der Telophase der Mitose für die lebende Zelle und das Zell-modell überein (Abb. 2).

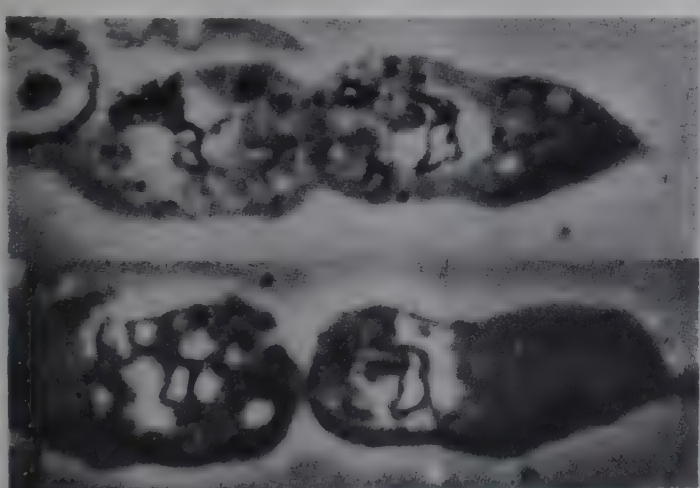


ABB. 2. — Zytokinese am Telophasemodell (Subcutisfibroblast) durch $2.5 \times 10^{-3}$ M ATP bei pH 7.4 und $I = 0.12$ Γ, 22° C. (nach Hoffmann-Berling, 24) 2 h. extrahierte Modelle; oben : ohne ATP; unten : 12 Min. nach ATP-Zugabe.

Und schliesslich beginnen auch die Modelle von Zellgeisseln auf ATP- und Mg-Zusatz ebenso zu schlagen wie lebende Geisseln (25). Dies wird besonders überzeugend sichtbar durch die kinematographische Darstellung (26). Die Kontraktion der Fasermodelle gleicht der Kontraktion der Muskeln, aus denen die Modelle hergestellt sind, quantitativ in der maximalen Spannung, der maximalen Verkürzung, der Abhängigkeit der Spannung von der Temperatur und in dem Nutzeffekt. mit dem chemische Energie in mechanische Arbeit umgesetzt wird (27, 28). Die Uebereinstimmung zwischen Modellkontraktion und Vitalkontraktion der gleichen Muskelart ist für die innere Gleichartigkeit der beiden Prozesse besonders beweisend, weil sie auch für solche Eigenschaften gilt, die von einer Muskelart zur andern ausserordentlich verschieden sind — wie z. B. die maximale Spannung oder die Temperaturabhängigkeit der maximalen Spannung. Qualitativ stimmt die Kontraktion des Fasermodells mit der Kontraktion des lebenden Ausgangsmaterials im Auftreten des quick-release-Phänomens (29, 30) und in der Tatsache überein, dass der Zusammenhang zwischen Belasting und Verkürzungsgeschwindigkeit auch für das Fasermodell durch die Hill'sche velocity-load-Gleichung dargestellt werden kann — wenn auch mit anderen Zahlenwerten für die Konstanten der Gleichung (31).

Der Unterschied in dem Zahlenwert der Geschwindigkeitskonstanten der Hill'schen Gleichung führt uns zu dem einzigen massiven Unterschied, der zwischen der Kontraktion der Fasermodelle und der lebenden Muskeln wenigstens dann besteht, wenn die Verkürzungsgeschwindigkeit der betreffenden Muskeln hoch ist. Denn in diesem Fall ist die Verkürzungsgeschwindigkeit der korrespondierenden Modelle etwa 10 × kleiner (32).

Diese einzige Verschiedenheit aber ist verständlich. Denn die Modelle verkürzen sich nur, wenn das Nukleosidtriphosphat von dem Fasermodell gespalten wird. Modelle aus schnellen Muskeln, wie den Skelettmuskeln der Säugetiere, aber spalten das ATP etwa 10 × langsamer als der lebende Muskel (27). Die geringere Verkürzungsgeschwindigkeit des Modells muss daher geradezu gefordert werden, wenn man annimmt, dass die ATP-Spaltung die Energie für die mechanische Arbeit liefert. Denn der andere Faktor der mechanischen Leistung — die Spannung — ist, wie schon gesagt, für die Modellkontraktion und die korrespondierende Vitalkontraktion gleich.

Wir sind offenbar berechtigt zu sagen, dass die experimentelle Isolierung des Kontraktionsvorganges mit Hilfe der Modelle den Kontraktionsvorgang häufig nur wenig oder gar nicht — und niemals qualitativ, sondern höchstens quantitativ — ändert.

## III

Alle diese isolierten Kontraktionsprozesse aber verlaufen im Prinzip gleichartig : Zellkontraktion wie Muskelkontraktion bedürfen 1) der Spaltung von ATP oder anderen Nukleosidtriphosphaten, 2) der Anwesenheit bestimmter $Mg^{++}$-Konzentrationen, deren Grösse nicht von der Art des Gewebes, sondern nur von der Art des NTP (Nukleosidtriphosphat) abhängt, 3) neutraler Reaktion.

Zellmodelle wie Muskelmodelle erschlaffen, wenn die ATP-Konzentration den Wert von $10^{-3}$ M überschreitet oder wenn die ATP-Spaltung vergiftet wird (27, 32).

Auch aus Zellen lassen sich die kontraktilen Proteine nach den gleichen Prinzipien extrahieren und reinigen wie aus Muskeln, da die Löslichkeit von kontraktilem Zell- und Muskelprotein in nahezu gleicher Art von Ionenstärke und ATP-Konzentration abhängt. Die Lösungen des isolierten kontraktilen Zellproteins zeigen auf ATP-Zusatz den gleichen reversiblen Viskositätsabfall wie Aktomyosinlösungen (Abb. 3). Die Gele schrumpfen unter ATP ebenso wie Aktomyosingele (Abb. 4) und spalten dabei gleichfalls das ATP (Tab. I). Damit stimmt

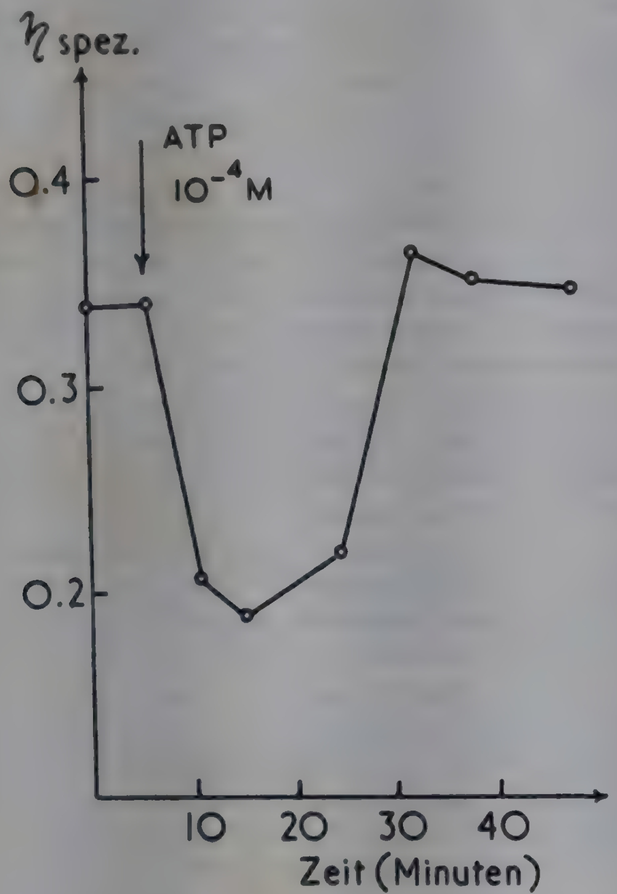


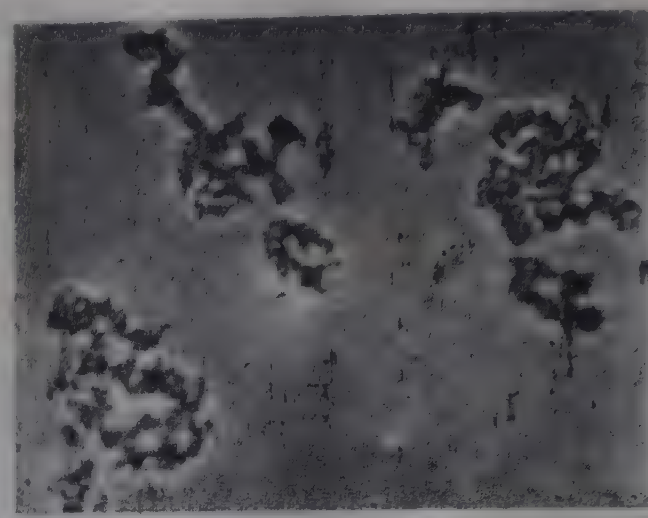

Abb. 3. — Viskositätsänderung einer Lösung von gereinigtem kontraktilem Zelleiweiss auf Zugabe von ATP (nach Hoffmann-Berling, 32*a*). Kontraktiles Protein isoliert aus Yoshida-Sarkomzellen, Ionenstärke = 0.6 Γ, 1 × $10^{-3}$ M $MgCl_2$, pH = 7, T = 22° C.

überein, dass auch die Flagellae von Zellen einerseits und Muskelfasern andererseits etwa das gleiche Röntgendiagramm geben (33).

Schliesslich findet sich auch die Weichmacherwirkung des ATP und anderer Phosphate bei allen kontraktilen Proteinen von der Einzelzelle bis zum höchstspezialisierten Muskel (32).

So lehrt uns das Studium der Modelle, dass alle vitalen Kontraktionen auf einer Reaktion des kontraktilen Proteins mit NTP beruhen, bei der das NTP gespalten wird und $Mg^{++}$ mitwirken.

Das bedeutet : ebenso wie der Betriebsstoffwechsel wird auch die Leistung mechanischer Arbeit durch einen Mechanismus ermöglicht, der im ganzen Bereich des Lebens gleich ist. Auch dieser Mechanismus ist von der Natur bereits auf der Entwicklungshöhe des Einzellers erfunden.



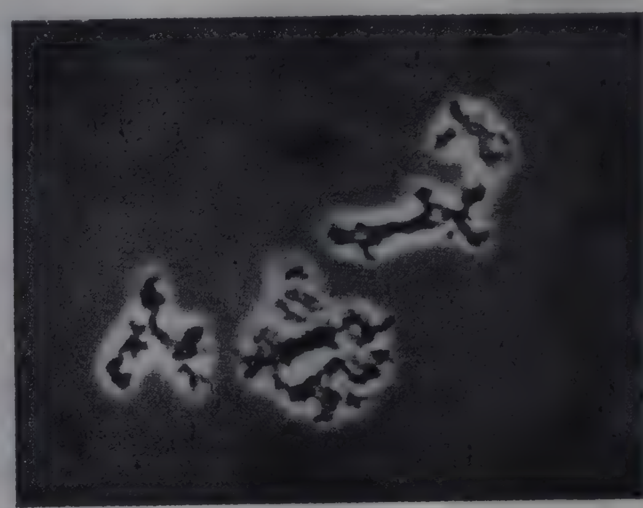


Abb. 4. — Schrumpfung der Flocken von isoliertem kontraktilem Zellprotein auf ATP-Zusatz (nach Hoffmann-Berling, 32*a*). Protein aus Yoshida-Sarkom : *a*) ohne ATP ; *b*) 60 Min. nach Zugabe von $10^{-3}$ M ATP, Ionenstärke = 0.09 Γ, pH = 7, T = 20° C.

Der weisse Hof um die Flocken in *b*) beruht auf der enormen Steigerung des Brechungsunterschiedes zwischen Flocke und wässriger Lösung als Folge der starken Zunahme der Proteindichte.

Tabelle I

*ATP-Spaltung von isoliertem kontraktilem Eiweiss aus Zellen und Muskeln (nach Hoffmann-Berling, 32a)*

| Art des Gewebes | Spaltung in $\frac{\mu\text{-Mol P}}{\text{mg. Min.}}$ | $\frac{\text{g kontraktiles Protein}}{\text{100 g Gewebe}}$ |
|---|---|---|
| Skelettmuskel . . . | 0.35 | ~10 |
| Zellen (Yoshida- und Jensen-Sarkom) | 0.003 | ~ 0.2 |

T = 20° C., Ionenstärke = 0.09 Γ, pH ~7

Dass die Spaltungsrate des kontraktilen Zellprotein nur etwa 1/100 der Spaltungsrate des Aktomyosin beträgt, dürfte sich daraus erklären, dass auch die Verkürzungsgeschwindigkeit — und damit die mechanische Leistung — der Zelle ausserordentlich viel kleiner (~1/1000) ist als die Verkürzungsgeschwindigkeit des Muskels (32).

IV

Nun sind im Jahre 1954 Arbeiten aus 2 Laboratorien erschienen (Fleckenstein, Janke, Davies and Krebs, 34, sowie Mommaerts, 35), nach denen sich unter Umständen während der Vitalkontraktion von Skelettmuskeln des Frosches die ATP-Konzentration nicht ändert und auch eine Restitution des ATP aus den bisher bekannten chemischen Reaktionen ausgeschlossen werden kann. Doch trifft dies nur zu, wenn die Arbeitsleistung sehr klein ist (34). Solche kleinen Arbeitsleistungen können erzielt werden durch kurze Tetanie bei 0° C. (34) oder durch Einzelreize bei tiefer Temperatur (35).

Für solche kleinen Arbeitsleistungen reicht also eine Energiequelle aus, die offenbar bisher unbekannt ist. Fleckenstein, Janke, Davies und Krebs finden bei solchen kleinen Arbeitsleistungen tatsächlich eine Abspaltung von Phosphat aus einer bisher unbekannten Muttersubstanz, die energetisch für die registrierte Arbeit ausreicht, wenn angenommen wird, dass dieses Phosphat aus einer energie-reichen Bindung abgespalten wird. Für die Frage nach dem Mechanismus der Muskelkontraktion sind diese Ergebnisse dann bedeutungsvoll, wenn dieser unbekannte Phosphat-Donator nicht der Restitution des ATP dienen sondern unmittelbar mit dem kontraktilen Protein der Muskeln und Zellen reagieren sollte.

Das erscheint zunächst durchaus möglich, weil die Spezifität der Reaktion zwischen kontraktilem Protein und phosphathaltigen Substanzen nicht sehr scharf ist :

— Alle anorganischen und organischen Polyphosphate machen die sonst rigiden kontraktilen Strukturen weich und setzen die Viskosität der Lösungen kontraktiler Proteine herab, falls genügend Mg zugegen ist.

— Aber auch alle untersuchten Nukleosidtriphosphate (dagegen die anderen organischen wie anorganischen Polyphosphate nicht) werden von den kontraktilen Strukturen von Muskel und Zelle im Modellversuch gespalten und bewirken gleichzeitig die Kontraktion dieser Strukturen, falls eine ausreichende Konzentration von Mg-Ionen vorhanden ist. An Nukleosidtriphosphaten sind bisher ausser ATP untersucht : Azetyl-ATP (36), ITP (Inosintriphosphat) (37, 23, 38, 36), GTP (Guanosintriphosphat) (39, 36) und UTP (Uridintriphosphat) (39, 36).

V

Die Wirksamkeit von ITP, UTP und GTP war bisher etwas umstritten. Dies beruht darauf, dass der Mg-Bedarf ihrer Reaktion mit dem kontraktilen Protein etwa 10 × grösser ist als der Mg-Bedarf der ATP-Reaktion. Infolgedessen verlaufen Spaltung und Kontraktion in ATP-Gegenwart auch ohne Mg-Zusatz mit ungefähr 2/3 der maximalen Intensität. Denn die immer vorhandene Konzentration von 0.5-1.0 × $10^{-5}$ M $Mg^{++}$, die nur durch besondere Reinigung weiter herabgesetzt werden kann, genügt für den Bedarf dieser Reaktion weitgehend (vgl. Tab. II und Abb. 5). Dagegen fehlt die Kontraktion durch ITP, UTP und GTP unter diesen Umständen vollständig (Tab. II) und die Spaltung fast vollständig (Abb. 5). Beide werden erst bei höheren Mg-Konzentrationen deutlich. Azetyl-ATP nimmt eine Mittelstellung zwischen ATP und den anderen Nukleosidtriphosphaten (NTP) ein (Tab. II und Abb. 5).

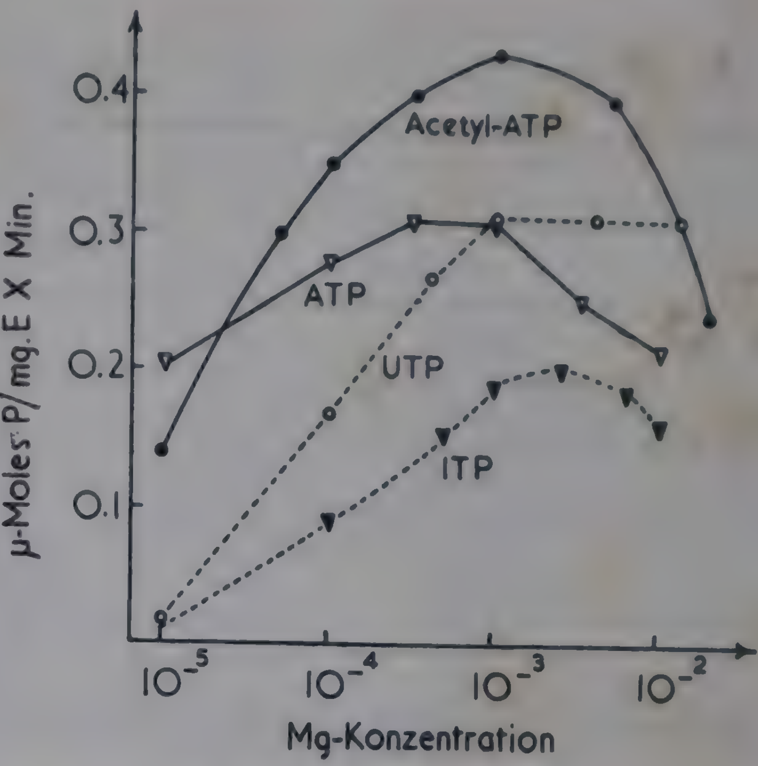



ABB. 5. — Die Abhängigkeit der Spaltung verschiedener Nukleosidtriphosphate (NTP) von der Mg-Konzentration : Aktomyosin (nach Hasselbach (36)). NTP-Konzentration 2 × $10^{-3}$ M, Ionenstärke = 0.1 Γ, pH = 7, T = 21° C.

Die gleiche Reihenfolge des Mg-Bedarfes gilt auch für die Weichmacherwirkung (Tab. III) der angeführten Nukleosidtriphosphate und — soweit untersucht — für die Minderung der Viskosität (Tab. IV) des gelösten Aktomyosin. Weichmacher- und Viskositätswirkung anorganischer Polyphosphate bedürfen einer Mg-Konzentration, die nochmals 1-2 Zehnerpotenzen höher ist als der Bedarf des ITP oder UTP.

Der Mg-Bedarf der verschiedenen Nukleosidtriphosphate hängt nur wenig ab von der Natur des kontraktilen Proteins, d.h. von der Frage, ob es sich um kontraktiles Zell- oder Muskelprotein handelt (38, 32). Dagegen hängt er deutlich ab von der Art und Funktion der Reaktion zwischen NTP und kontraktilem Protein. Denn für alle Nukleosidtriphosphate ist der Mg-Bedarf der NTP-Spaltung und NTP-Kontraktion deutlich höher als der Mg-Bedarf der Weichmacher- und der Viskositätswirkung, für die er ungefähr gleich ist (vgl. Tab. II und Abb. 5 mit Tab. III u. Tab. IV).

Ueber den Mg-Bedarf ergibt sich demnach zusammenfassend :

*a*) Er wächst in der Reihenfolge ATP < Azetyl-ATP < ITP ≅ GTP ≅ UTP.

*b*) Er ist für Weichmacher- und Viskositätswirkung kleiner als für Spaltung und Erzeugung mechanischer Spannung.

VI

Die unter (*b*) angeführte Tatsache spricht dafür, dass die in ihrer Bedeutung viel umstrittene ATP-Wirkung auf die Viskosität von gelöstem Aktomyosin verwandt ist mit der Weichmacherwirkung auf das Aktomyosingel — und nichts zu tun hat mit der Kontraktion. Hierfür spricht auch die Tatsache, dass die Weichmacher- und Viskositätseffekte durch Senkung der Temperatur begüns-

TABELLE II.

*Der Mg-Bedarf der aktiven Spannungen in Abhängigkeit von der Art der kontraktionserzeugenden Nukleosidtriphosphate (nach Hasselbach, 36)*

| Mg-Konzentration | Spannungen in % der Maximalspannung unter ATP erzeugt durch | | | | |
|---|---|---|---|---|---|
| | ATP | Azetyl-ATP | ITP | GTP | UTP |
| ~$10^{-11}$ M (in Gegenwart von EDTA) | ~10 | 0 | — | — | — |
| <$10^{-5}$ M (nach Vorbehandlung mit EDTA) | ~15 | — | — | — | — |
| ~$10^{-6}$ M (normale Modelle, nicht mit EDTA vorbehandelt) | ~60 | 30 | 0 | 0 | 0 |
| $10^{-4}$ M | — | — | ~10 | — | — |
| $10^{-3}$ M | 80-100 | — | — | ~20 | — |
| 5 × $10^{-3}$ M | 100 | 70 | 60 | — | ~75 |
| $10^{-2}$ M | 100 | — | 60 | ~50 | — |

Fasermodelle aus Kaninchenpsoas ; pH = 7 ; Ionenstärke ~0.1 Γ ; NTP-Konzentration 3-6 × $10^{-3}$ M ; Ausgangslänge der Fasermodelle ~80 % der Standardlänge ; T = 20° C.

TABELLE III.

*Mg-Bedarf des Weichmachereffektes verschiedener Polyphosphate bei Hemmung der NTP-Spaltung und Kontraktion (nach Hasselbach, 36)*

| Mg-Konzentration | Elastizitätsmodul in % des Elastizitätsmoduls in Abwesenheit aller Polyphosphate nach Zusatz von | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ATP | | Azetyl-ATP | ITP | | UTP | Pyrophosphat | |
| | 0° C. | 20° C. | 0° C. | 0° C. | 20° C. | 0° C. | 0° C. | 20° C. |
| ~$10^{-11}$ M (*) | 18 | 25 | — | 30 | 100 | — | 100 | 100 |
| <$10^{-5}$ M (*) | ~7 | ~7 | — | — | ~60 | — | — | — |
| ~$10^{-6}$ M (*) | ~4 | ~4 | 30 | 15 | 30 | ~40 | 100 | 100 |
| $10^{-3}$ M | ~4 | ~4 | — | 10 | — | — | 20 | 50 |
| 5 × $10^{-3}$ M | 2.5 | 2.5 | 15 | 10 | — | ~20 | 10 | 20 |
| $10^{-2}$ M | 2.5 | 2.5 | — | 7 | — | — | 10 | 20 |

(*) Hergestellt wie in Tabelle II.
Fasermodelle aus Kaninchenpsoas ; pH = 7 ; Ionenstärke 0.1Γ ; Polyphosphat-Konzentration = 5 × $10^{-3}$ M.

TABELLE IV.

*Mg-Bedarf der Viskositätseffekte verschiedener Polyphosphate (nach Hasselbach, 36)*

| Mg-Konzentration | Viskositätsabfall in % der maximalen ATP-Empfindlichkeit auf Zusatz von | | | | | |
|---|---|---|---|---|---|---|
| | ATP | | ITP | | Pyrophosphat | |
| | 0° C. | 20° C. | 0° C. | 20° C. | 0° C. | 20° C. |
| ~$10^{-11}$ M (*) | 100 | 0 | 0 | 0 | 0 | 0 |
| ~$10^{-5}$ M (*) | — | ~75 | — | — | — | — |
| ~2 × $10^{-5}$ M (*) | 100 | 100 | — | ~15 | ~25 | 0 |
| $10^{-3}$ M | 100 | 100 | — | 100 | 100 | 50 |
| $10^{-2}$ M | 100 | 100 | — | 100 | 100 | 100 |

Aktomyosinlösung aus Kaninchenmuskeln ; Ionenstärke = 0.6Γ ; pH = 7 ; Polyphosphatkonzentration 1 × $10^{-3}$ M.

(*) Hergestellt wie in Tabelle II, doch sind die Konzentrationen in den Lösungen ohne Mg-Zusatz dann höher, wenn kein Enthärter zugegen ist, weil 0.6 M KCl-Lösungen deutliche Spuren von Mg enthalten.

tigt werden (vgl. Tab. III und IV), während ATP-Spaltung und — Kontraktion mit fallender Temperatur kleiner werden (z. B., 28). Offenbar setzen die Nukleosidtriphosphate die Kohäsion des Aktomyosinkomplexes herab, wodurch im Gel die Rigidität schwindet (Weichmacherwirkung) und im Sol die Viskosität vermindert wird.

Dass der gelöste Aktomyosinkomplex tatsächlich unter ATP in Aktin und Myosin dissoziiert, scheint durch die Ergebnisse von A. Weber (New York, 40) bewiesen : Homogene Aktomyosinlösungen lassen sich nach ATP-Zusatz mit der Ultrazentrifuge in reines L-Myosin und weitgehend reines Aktin zerlegen. A. Szent-Györgyi's Dissoziations-theorie der ATP-Wirkung auf gelöstes Aktomyosin (12*a*, 41) scheint trotz der Einwendungen von Morales *et al.* (42) richtig zu sein.

Vor allem aber zeigt die unter (*b*) berichtete Tatsache, dass das Mg an mindestens 2 Gliedern der Reaktionskette zwischen NTP und kontraktilem Protein teilnimmt : in geringerer Konzentration ermöglicht das Mg die Bindung der Polyphosphate an das Protein, auf der die Weichmacherund Viskositätswirkung beruht, in höherer Konzentration dagegen wird es benötigt für den späteren Schritt der Spaltung und Kontraktion.

Dagegen lässt der unter (*a*) berichtete verschieden grosse Mg-Bedarf der verschiedenen Nukleosidtriphosphate keine Entscheidung zu, welches NTP während der physiologischen Kontraktion bevorzugt wird. Denn die physiologische Mg-Konzentration ist mindestens im Muskel so hoch, dass sie dem Mg-Bedarf auch des anspruchsvollsten NTP genügt.

## VII

Nun aber zeigt Tab. II, dass die Spannungsentwicklung von Muskelmodellen unter der Einwirkung der verschiedenen Nukleosidtriphosphate auch dann verschieden gross ist, wenn die Mg-Konzentration ($0.5\text{-}1.0 \times 10^{-2}$ M) hoch genug ist, um die Kontraktion durch alle Nukleosidtriphosphate voll zu aktivieren. Auch in diesem Fall erzeugt ATP eine wesentlich höhere Spannung als die anderen Triphosphate. Da das gleiche für die Spaltung nicht gilt (Abb. 5), sieht es so aus, als sei der Nutzeffekt bei Spannungserzeugung durch ATP besonders hoch. Das aber wäre ein teleologisches Argument für die Bevorzugung des ATP während der physiologischen Kontraktion.

Dieses Argument wird unterstützt durch folgende Beobachtung :

Wird zu Fibrillenmodellen, aus denen das Nudiki (Nukleosiddiphosphokinase) vollständig ausgewaschen ist, eine Mischung von radioaktiven ATP mit inaktivem ITP und IDP gegeben, so bleiben nicht nur die ITP- und IDP-Konzentrationen unverändert, bis das ATP nahezu erschöpft ist, sondern es wird ausserdem auch kein radioaktives Phosphat auf das IDP oder ITP transphosphoriliert (43). Dieser Befund zeigt, dass solche Fibrillenmodelle ITP überhaupt nicht angreifen, solange der ATP-Spiegel nicht auf sehr niedrige Werte abgesunken ist. Infolgedessen scheidet das ITP als Betriebsmittel der Muskelkontraktion aus, solange ATP vorhanden ist. Doch fehlen analoge Versuche mit den übrigen Nukleosidtriphosphaten.

## VIII

Nun besteht der Kontraktionszyklus nicht nur aus der Kontraktionsphase, sondern auch aus der Erschlaffungsphase. Aktomyosinstrukturen aber scheinen unter physiologischen Verhältnissen nur zu erschlaffen, wenn der von Marsh unter Bailey entdeckte (44) und von Bendall (45) weitgehend gereinigte Faktor in aktivem Zustand zugegen ist (*). Es ist schon länger bekannt (47), dass der Marsh-Faktor die ATP-Spaltung und ATP-Kontraktion dadurch unterdrückt, dass er die Grenze der überoptimalen ATP-Konzentration herabdrückt. Infolgedessen werden ATP-Spaltung und ATP-Kontraktion durch den Marsh-Faktor nicht unterdrückt, sobald die ATP-Konzentration wesentlich niedriger ist als die physiologische Konzentration. Man kann also die Wirkung des Marsh-Faktors auf die verschiedenen Nukleosidtriphosphate auch dadurch charakterisieren, dass man die niedrigste NTP-Konzentration angibt, bei der der Marsh-Faktor die Spannungsentwicklung aufhebt. Es zeigt sich (Tab. V), dass die durch ITP, GTP und UTP bewirkte Spannungsentwicklung durch aktiven Marsh-Faktor bis zu einer NTP-Konzentration von $10^{-2}$ M überhaupt nicht beeinflusst wird, während die Spannungsentwicklung durch Azetyl-ATP nur aufgehoben wird für Konzentrationen von $7.5 \times 10^{-3}$ M an aufwärts. Die angegebenen Zahlen bedeuten, dass überhaupt nur ATP im Muskel in einer Konzentration vorhanden ist, in der Spaltung und Kontraktion durch Aktivierung des Marsh-Faktors beeinflusst wird. Wenn GTP, UTP und ITP trotz ihrer minimalen Konzentration im Muskel überhaupt eine Kontraktion hervorrufen könnten, dann sollte diese Kontraktion eine Dauerkontraktion

TABELLE V

*Die Wirkung des Marsh-Faktors auf die Spannungsentwicklung durch verschiedene Nukleosidtriphosphate*
*(nach Hasselbach, 36)*

| Art des NTP | Minimalmolarität des NTP, in der der Marsh-Faktor die Spannungsentwicklung aufhebt |
|---|---|
| ATP | $2.5 \times 10^{-3}$ |
| Azetyl-ATP | $7.5 \times 10^{-3}$ |
| ITP | bis $10^{-2}$ nicht |
| GTP | bis $10^{-2}$ nicht |
| UTP | bis $10^{-2}$ nicht |

Fasermodell aus Kaninchenpsoas, pH = 7,
Mg-Konzentration = $10^{-2}$ M, Ionenstärke = 0.16 Γ, T = 20° C.

(*) Die Wirkung dieses Faktors beruht nicht einfach auf einer Umphosphorylierung von Kreatinphosphat auf ADP, wie gelegentlich behauptet worden ist (vgl. das folgende Referat von S. V. Perry, Cambridge). Dass der Faktor nicht einfach Phosphokreatin + Phosphopherase ist, geht sowohl aus Bendall's Reinigungsexperimenten (45) wie auch daraus hervor, dass der Faktor die Spaltung physiologischer ATP-Konzentrationen hemmt. Denn das System Phosphokreatin + Phosphopherase hemmt nach neueren Untersuchungen (46) die ATP-Spaltung der Modelle nie.

sein und der Muskel ausserstande sein, zwischen Kontraktion und Erschlaffung zu wechseln.

Wenn also der neue Phosphatdonator mit dem kontraktilen Protein unmittelbar reagieren sollte, dürfte er keines der bereits bekannten Nukleosidtriphosphate sein. Er müste vielmehr in seinem Verhalten viel ATP-ähnlicher sein als GTP, ITP, UTP und selbst Azetyl-ATP.

## IX

Der molekulare Mechanismus, durch den die Spaltungsenergie des Nukleosidtriphosphats auf das kontraktile Protein übertragen und in mechanische Arbeit und Wärme verwandelt wird, dürfte von der speziellen Natur des verwendeten Nukleosidtriphosphats wahrscheinlich einigermassen unabhängig sein.

Wir wissen aus unserer Kenntnis des Betriebsstoffwechsels, dass überall dort, wo die Energie einer sogenannten « energiereichen » Phosphatbindung übertragen wird, diese Uebertragung durch eine Umphosphorylierung erfolgt. Wir wissen ferner, dass die grosse Zahl der bekannten Umphosphorylierungen auf hohem Energieniveau immer des Mg bedürfen und dass sehr häufig Sulfhydrilgruppen dafür notwendig sind.

Wir wissen schliesslich durch Lynen, Lipmann und Mitarbeiter (48, 49), dass in einigen Fällen, in denen ebenfalls durch eine ATP-Hydrolyse Arbeit — und zwar chemische Arbeit — geleistet wird, in Wirklichkeit eine Kettenreaktion vorliegt. Die Reaktionskette beginnt in diesem Falle mit einer Phosphorylierung des beteiligten Enzym-Proteins.

Die Kontraktion aller kontraktilen Proteine erhält ihre Energie durch einen Vorgang, der in der Bilanz wie eine NTP-Hydrolyse aussieht. Die Reaktion bedarf des Mg und der SH-Gruppen des kontraktilen, d.h. des enzymatisch wirksamen Proteins.

Es liegt also nahe, auch hier als ersten Schritt der Reaktionskette eine « energiereiche » Phosphorylierung des kontraktilen Proteins durch Nukleosiddiphosphat (NDP) oder Phosphat anzunehmen. Die Verwandlung der so auf das Protein übertragenen Energie in mechanische Arbeit und Wärme durch die weiteren Glieder der Reaktionskette müsste sich dann auf dem Teilchen des kontraktilen Proteins selbst vollziehen.

Alle Versuche, ein solches Reaktionsschema durch Beobachtung der Rückreaktion des ersten Schrittes, d.h. durch die Rückreaktion der Umphosphorylierung auf das kontraktile Protein zu beweisen, sind bisher gescheitert (Lipmann, 50; Koshland, 51, und unsere eigenen Versuche, 43) : denn es gelang bisher nie, in ATP $^{32}$P einzubauen, wenn kontraktiles Protein das ATP in Gegenwart von radioaktiven Phosphat oder radioaktivem ADP spaltete. Das ist zunächst nicht erstaunlich. Denn anders als bei der Azetylierung des Coenzym A (48) oder der Synthese der Pantothensäure (49) mit Hilfe der Spaltungsenergie des ATP lässt sich die Reaktionskette auf dem kontraktilem Protein nicht gezielt unterbrechen. Wenn infolgedessen die weiteren Reaktionen dem ersten Schritt sehr schnell folgen, wird die Wahrscheinlichkeit der Rückreaktion des ersten Schrittes sehr klein. Ein schnelles und irreversibles Weiterreagieren aber ist wahrscheinlich, weil der Energiegehalt des kontraktilen Komplexes dabei stark absinkt. Gewisse Argumente für schnelles Weiterreagieren nach dem ersten Schritt sind auch von Mommaerts beigebracht (52).

Ausserdem ist die Wahrscheinlichkeit für das radioaktive ATP, das durch eine etwaige Rückreaktion gebildet ist, sofort wieder gespalten zu werden, viel grösser als für das inaktive ATP. Denn das radioaktive ATP befindet sich im Augenblick seiner Entstehung bereits am Reaktionsort im Innern des kontraktilen Gels ; das inaktive ATP dagegen muss in das Gel erst eindiffundieren.

Es ist uns aber auch nicht gelungen, eine Rückreaktion dann zu finden, wenn die ATP-Spaltung durch Mg-Mangel, SH-Reagenzien wie Salyrgan oder Polysulfosäuren wie Germanin vergiftet war. Da aber diese Vergiftungsversuche ungezielt sind, so dass man nicht weiss, ob die Reaktionskette unmittelbar hinter dem Umphosphorylierungsschritt unterbrochen wurde oder aber später oder gar vorher, beweisen diese negativen Ergebnisse nicht allzu viel.

Auf der anderen Seite aber liefert das vorgeschlagene Reaktionsschema sofort auch die Möglichkeit, die mit der Spaltung gekoppelte Kontraktion zu verstehen. Dies geht schematisch und in den Einzelheiten willkürlich aus Abb. 6 hervor. Die Abbildung 6 zeigt, wie die Reaktion der

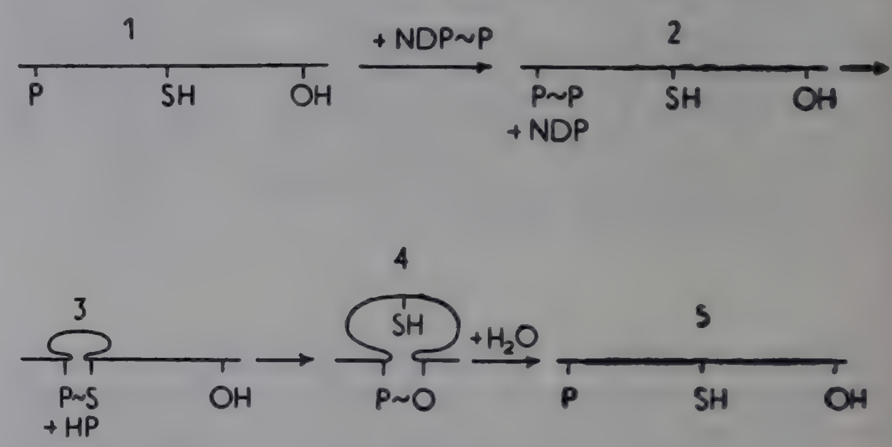



Abb. 6. — Schema der Koppelung von NTP-Spaltung mit Verkürzung des kontraktilen Protein durch Umphosphorylierung und anschliessende Kettenreaktion der funktionellen Gruppen des Protein. Zustand **1** vor Zugabe des NTP ; Zustand **2** nach Umphosphorylierung ; Zustand **3** und **4** weitere Glieder der Kettenreaktion ; Zustand **5** nach Ende der Reaktion.

Gruppen, die an der Reaktionskette beteilkigt sind, zu einer Verkürzung der Peptidkette des kontraktilen Eiweisses führt, falls man der Peptidkette eine gewisse thermokinetische Beweglichkeit zubilligt (vgl. dazu Buchtahl, 53).

Aus der Abbildung 6 wird aber auch verständlich, dass die Kettenreaktion umso schneller ablaufen muss, je näher die reagierenden Gruppen aneinander sitzen. Das heisst : wenn eine Peptidkette passiv verkürzt wird, weil sich die Nachbarkette aktiv verkürzt hat, wird die Kettenreaktion in dieser Kette schneller ablaufen. Umgekehrt sollte es durch starken Zug an der Peptidkette möglich sein, etwa den Zustand **3** in den Zustand **2** zurückzuverwandeln, wenn die Peptidkette durch Zug von aussen gestreckt wird.

Das vorgeschlagene Reaktionsschema macht also nicht nur verständlich, wie die Peptidkette durch den chemischen Umsatz verkürzt wird, sondern auch dass umgekehrt Verkürzung und Streckung der Peptidkette

den chemischen Umsatz herauf- und herabsetzen. Hierdurch aber entstehen Beziehungen — wenn auch durchaus noch in unzulänglicher Form — zu der von Hill beobachteten Steigerung des isometrischen Umsatzes durch Verkürzung und Umsatzverminderung durch Streckung des Muskels (54). Dies alles mag als ermutigend angesehen werden.

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## Nachtrag

(*Eingegangen am* 13 *September* 1955)

Die Vorlesung auf dem Kongress in Brüssel am 2. August 1955 unterscheidet sich von den oben mitgeteilten, bereits im April eingesandten Ergebnissen in folgenden zwei Punkten :

(*a*) Die Vermehrung der untersuchten Nukleosid-triphosphate (NTP) deckte gewisse Unterschiede auf in der Reihenfolge der NTP-Wirkung auf die NTP-Spaltung und Spannungsentwicklung einerseits und auf die Weichmacherwirkung andererseits. Die maximale Spannung und Spaltungsgeschwindigkeit nehmen ab vom ATP zum GTP in der Reihenfolge :

$$\text{ATP} > \text{Acetyl-ATP} \sim \text{CTP (Cytidintriphosphat)} > \text{UTP} >> \text{ITP} >> \text{GTP}$$

Dagegen nimmt die Weichmacherwirkung ab vom ATP zum CTP in der Reihenfolge :

$$\text{ATP} > \text{GTP} > \text{ITP} > \text{Acetyl-ATP} > \text{UTP} = \text{CTP}$$

Die abnehmende Grösse der Wirkung in den angeführten beiden Reihen der NTP's ist verbunden mit zunehmendem Mg-Bedarf der betreffeden NTP's. Die angeführten Reihen zeigen, dass die Verwendbarkeit der verschiedenen NTP's für Spaltung und Spannung mehr von der Aminogruppe in 6-Stellung (*) abhängt als von der Konstitution des Ringes als Purinring oder Pyrimidinring. Im Gegensatz hierzu ist die Weichmacherwirkung aller Purinderivate (unabhängig von der Gegenwart einer $NH_2$-Gruppe in 6-Stellung) für alle Purinderivate deutlich grösser als für die Pyrimidinderivate.

(*b*) Inzwischen wurde der Phosphataustausch zwischen ATP und ADP gefunden, der als Voraussetzung der Annahme gefordert werden muss, dass die Spaltung und die Kontraktion durch eine Transphosphorylierung des endständigen Phosphats des ATP auf das Aktomyosin eingeleitet werden. Wird $AD^{32}P$ und ATP mit 10-fach gewaschenen Muskelfibrillen zusammengebracht und die ATP-Spaltung durch « Fuadin » (Antimon-*bis*-Brenzkatechindisulfonat) zu 90 % gehemmt, so wird ein Ausgleich des $^{32}P$ zwischen ATP und ADP erreicht. Die Fibrillen sind frei von Nudiki und enthalten so wenig Myokinase, dass die Bildung von $AT^{32}P$ durch Myokinase nur etwa ein Viertel der Produktion von $AT^{32}P$ ausmacht.

(*) Acetyl-ATP ist in der Ribose 2-fach und nicht in der Aminogruppe acetyliert.

# Nucleotide metabolism and intracellular organisation in skeletal muscle

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(*Received* 21 *April* 1955)

In view of the quantitative contribution of actin and myosin to the total material found in the myofibril and the unique nature of their interaction with ATP (*) and each other, these proteins are of undisputed significance in the contractile process. The precise role of each protein and the way they are oriented with respect to one another in the contractile element is still far from understood, although recently some progress has been made in this direction. If we accept the view that the dephosphorylation of ATP is an essential requirement for contraction, then in so far as the nucleotide is a substrate for the enzymic activity of myosin, it is a natural step to consider that this protein is instrumental in utilising the energy of the phosphate bond for the performance of mechanical work. Under physiological conditions actin appears to be necessary for this process but as yet there is little precise evidence as to how this interconversion is accomplished. As regards contraction there are suggestions from the work of Kafiani and Engelhardt (1) that its role may be a passive one, for fibres made from surface films of actin-free myosin (2) will contract anisodimensionally at pH 9 in the presence of ATP. On the basis of this result and that obtained by Ashmarin (3) who was able to demonstrate similar contraction effects at pH 7 if the myosin was first treated with certain dyes, it seems that the role of actin may be to modify the charge on the myosin so that the contraction can take place at physiological pH values. Precise orientation of actin and myosin with respect to one another does not appear to be important so far as contraction alone is concerned for fibres made from precipitated actomyosin will shorten to the same extent as isolated myofibrils or glycerated fibres. Nevertheless very much greater tensions on the addition of ATP are obtained with the latter system (4), and it seems that the orientation of actin with respect to the myosin filaments as in the integrated system of the myofibril is essential for the development and transmission of tension.

(*) Abbreviations used : ATP = Adenosinetriphosphate ; ADP = Adenosinediphosphate ; ITP = Inosinetriphosphate ; IDP = Inosinediphosphate ; UTP = Uridinetriphosphate ; UDP = Uridinediphosphate ; GTP = Guanosinetriphosphate ; DNP = 2 : 4 Dinitrophenol ; PMA = Phenyl mercury acetate ; Versene = Ethylenediamine tetraacetate.

## PROTEIN COMPONENTS

Ultimate understanding of the nature of the contractile process demands a knowledge of the fine structure of the myofibril in terms of its constituent proteins. An important advance in this direction has been made by the work of Hasselbach (5) and Hanson and Huxley (6) who have demonstrated that the A band can be selectively removed from myofibrils by salt solutions which extract myosin free from actin. The results suggest that at least the majority of myosin is localised in the A band and that actin is the main component of the filaments which remain after the removal of myosin and which appear to run continuously along the length of the sarcomere. On the basis of earlier cross section studies (7) and the results of selective extraction it has been suggested (8, 9) that actin and myosin are laid down in separate filaments of 40 Å and 110 Å diameter respectively. It is now clear that moderate degrees of isotonic contraction (up to 30 %) involve changes in length of the I rather than the A band, which means on the basis of this model that the actin filaments must shorten, whereas the conventional view has usually attributed this property to myosin or actomyosin. If the myofibril consists simply of these two types of filaments it is unlikely that either will be homogeneous with respect to protein type for there is increasing evidence that an appreciable part of the myofibril consists of proteins other than actin and myosin.

Of these tropomyosin (10, 11) is by far the best characterised and accounts for 4 % of the total proteins of the rabbit skeletal myofibril (12). Certain similarities between the amino acid composition of tropomyosin and the other myofibrillar proteins have been noted by Bailey and he has suggested that the former may be a precursor of myosin. In this respect it is of interest that Kominz *et al.* (13) have shown that a mixture of equal amounts of actin and tropomyosin has a remarkably similar amino acid composition to myosin.

By prolonged extraction at pH 7.1 with 0.078 M borate buffer tropomyosin can be extracted from the myofibril together with another protein which will be referred to at this stage as the myofibrillar pseudoglobulin. The borate extract has a rather high viscosity, which so far as can be judged from the properties of the mixture is due entirely to the tropomyosin component for the addition of 0.2 M KCl reduces the viscosity to a very low level (12). The pseudoglobulin has no ATPase activity, is not G or F-actin and although it was suspected that it might be some form of depolymerised actin the true nature of this substance has not yet been determined (see addendum). When examined in the Tiselius electrophoresis apparatus at pH 6.7 and $\mu = 0.34$ the pseudoglobulin moves slower than the tropomyosin component and if actin, depolymerised by previous treatment with KI, is added to the extract it moves as a separate peak between the tropomyosin and pseudoglobulin components (14).

After prolonged extraction of rabbit myofibrils at 0° C. the solution obtained contains about 13 % of the total myofibrillar protein; of this tropomyosin amounts to about 4 % and the remainder representing 8-9 % of the total myofibrillar protein, consists of the pseudo-

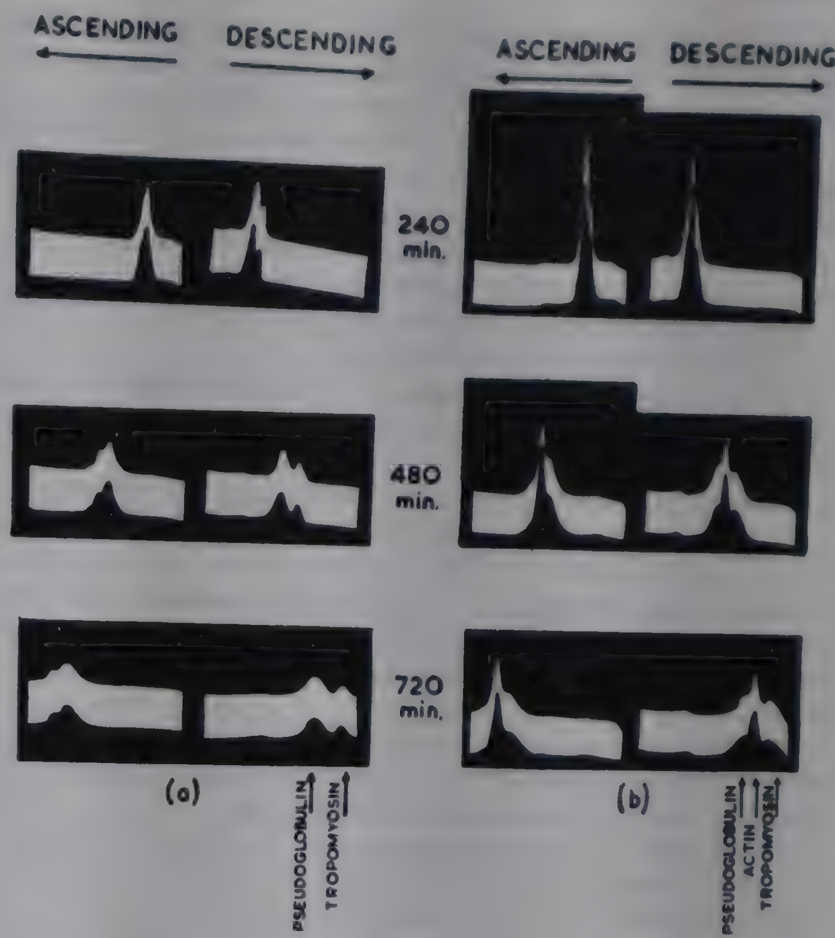


FIG. 1. — Electrophoretic diagrams of the protein solution obtained by extracting isolated rabbit myofibrils with 0.078 M borate buffer pH 7.1, for 10 days at 0° C. The depolymerised actin added to the extract was obtained by treating-F-actin with 1.2 M KI. Composition of buffer used for electrophoresis 0.25 M KCl, 0.05 M phosphate buffer pH 6.7. (*a*) Borate extract; (*b*) Borate extract + depolymerised actin.

globulin. When such borate-extracted myofibrils were examined with the electron microscope they appeared to have had material extracted from the centre of the A band as a result of which the outer edges of this band appeared very prominent (12, 15).

More recently A. G. Szent-Györgyi *et al.* (16) have found that when washed homogenised glycerated fibres are extracted with the solutions used by Hasselbach (5) and Hanson and Huxley (6) to remove the A band, considerable amounts of protein other than myosin pass into solution. The fractionation carried out by these workers suggests that soluble proteins other than myosin and actin constitute about 20 % of the myofibrillar proteins, which figure is rather higher than that obtained earlier by Perry (12, 15). Although detailed study of this non-myosin fraction has not been reported no doubt in part it consists of the myofibrillar pseudoglobulin and possibly tropomyosin. Although most of the protein must be of myofibrillar origin some may be derived from other structures because in the preparation of myofibrils from glycerated fibres no mention is made of the removal of nuclei and cell debris which sediment with the myofibrillar fraction.

There is some evidence also that the myofibril contains in addition to those described above, less soluble proteins for although Weber's solution or solutions containing KI bring 96-98 % of the total protein into solution, repeated extraction at 0° C. with LiCl, which is an extremely efficient solvent for actomyosin (17), leaves behind a

residue representing about 9 % of the total protein. This fraction probably contains proteins derived from cross striae such as the Z band etc. and possibly from non-contractile longitudinal elements of purely structural function. The presence of such elements is suggested from the behaviour of isolated myofibrils made to contract while fixed in a fibrin-clot (18) for under these conditions the contractile material shortens within what appears to be a framework of fibrous material which maintains the general shape of the myofibril.

In addition to the above proteins which have been obtained by extraction of the isolated myofibril, Dubuisson has identified two other components, namely contractin and the Y-protein which can be considered to be of myofibrillar origin because they are obtained only by extraction of whole muscle slices with solutions of high ionic strengths. Both these proteins undergo changes in extractability in activity with the result that the yields obtained are determined by the physiological state of the muscle.

Contractin appears as a very small peak which moves behind the myosin component on electrophoresis of extracts made from resting muscle (19, 20). If the muscle is contracted by monobromoacetate, or fixed in the stimulated state by plunging into liquid air, the contractin peak increases in amount whereas the myosin component disappears and the actomyosin is much reduced. It appears that contractin is identical with the γ-myosin component which is usually found in Weber-Edsall myosin preparations (21). The Y-protein (22) requires extraction by solutions of high ionic strength to bring it into solution but in contrast to contractin, if KCl is used as the extractant it can only be obtained from extracts of resting muscle. Nevertheless when extraction is carried out with solutions containing KI or pyrophosphate the Y-protein is obtained also from muscle in rigor or contracture. The Y-protein has been partially purified by ammonium sulphate precipitation and has globulin properties.

It is clear that in addition to the well characterised myosin, actin and tropomyosin, the myofibril contains several other proteins (table I) and any explanation of myofibrillar structure simply on the basis of actin and myosin is inadequate. The relation between the less well-characterised myofibrillar proteins which various workers have described is not clear. The pseudoglobulin of Perry, Dubuisson's Y-protein and the non-myosin fraction of A. G. Szent-Györgyi have certain similarities in that all are soluble at low ionic strength and their solutions are not particularly viscous. It is dangerous to take this comparison too far for only the pseudoglobulin was extracted from purified myofibrils. No doubt this fraction has escaped notice for it tends to precipitate with actomyosin on reducing the ionic strength to 0.04. From time to time, however, investigators have reported the presence of other proteins in purified myosin preparations which very likely consisted of such minor components of the myofibril (27, 28). Dubuisson (20) and Weber and Portzehl (4) have suggested a number of possible explanations for the appearance of the contractin peak in extracts of stimulated muscle but until further information is available we must at this stage conclude with the latter authors that contraction involves protein reactions of which we have as yet little cognizance.

Although they constitute the bulk of muscle tissue the myofibrils are deficient in general metabolic activity, for

TABLE I

*The protein components of the myofibril*

| Protein component | Molecular weight | Localisation | Remarks | Author |
|---|---|---|---|---|
| Myosin | 850 000 | A band | | |
| Actin | 70 000 (G monomer) | I and A bands | | Straub (24, 25) |
| Tropomyosin | 53 000 (monomer) | | | Bailey (10, 11) |
| Y-protein | | | Less readily extracted from rigor or contracture muscle. | Dubuisson (22) |
| Pseudoglobulin | | | Depolymerised actin ? | Perry and Corsi (12, 14) |
| Non-myosin fraction | | A band ? | | A. G. Szent-Györgyi *et al.* (16) |
| Contractin (γ-myosin) | | | Increases in amount in extracts from contracted muscle. | Dubuisson (19, 20) |
| N-protein | | I band | Existence doubtful as is based on indirect evidence (23). | Gerendas and Matoltsy (26) |
| LiCl insoluble residue | | | Cross striae and longitudinal structural elements ? | Perry (12) |

most of the enzymic activity of the cell is associated with the sarcoplasm and sarcosomes which represent about one third of the total protein of skeletal muscle. In contrast to those of the myofibril the sarcoplasmic proteins are in the sol form and although it is the less viscous part of the cell the sarcoplasm may be more concentrated with respect to protein (9). Recent investigations have indicated that the classical fractions obtained from sarcoplasmic extracts namely globulin X, Myogen A and B usually contain several components when they are examined by the Tiselius electrophoresis method (29-32). It seems that practically all the myogen components suffer some denaturation on dialysis and the globulin X consists of these denatured myogens together with the sarcoplasmic globulins, and any sarcoplasmic granules which were present in the original extract would probably aggregate on dialysis and precipitate in this fraction. Although slight individual variations are found (31) the electrophoretic pattern of sarcoplasm is characteristic of the species and between species very considerable variation in the relative amounts of the components are found (29). In rabbit skeletal sarcoplasm eleven components have been recognised by Jacob (33) and some attempt has been made to relate these to known enzymes of the glycolysis system. This may be possible with the enzymes such as triosephosphate dehydrogenase and aldolase which are present in relatively large amounts, but in view of the fact that there are probably some 50-100 different enzymes in sarcoplasm few if any of the peaks seen in the electrophoretic diagrams represent individual proteins. It is evident from Marsh's original study (34) as well as from subsequent investigations that the relaxing factor must also be a sarcoplasmic constituent because it can be readily extracted by solutions of low ionic strength in which the myofibril is insoluble. This means that the whole of the ATP metabolism of the muscle cell is under sarcoplasmic control. ATP synthesis is brought about anaerobically by the soluble glycolytic enzymes and aerobically by the sarcosomes, whereas the main system of ATP catabolism, the myofibrillar ATPase, is controlled by the relaxing factor which is likewise distributed in the sarcoplasm.

## NUCLEOTIDE METABOLISM

Almost without exception *in vitro* studies with contractile models provide convincing evidence that shortening is accompanied by the liberation of inorganic phosphate from ATP. It is not intended here to discuss whether this is a necessary consequence of the contractile phase of the process, but rather to consider some properties of the myofibrillar ATPase system. Although in the early days of the discovery of the ATPase activity of myosin it was widely considered that ATP was the direct source of the inorganic phosphate which accumulates during muscle activity, in latter years acceptance of this view has been brought into question (35-38).

Caution in the acceptance of this hypothesis has been justified by recent investigations (39-43) on the changes in the nucleotide level during a single twitch. The results of these investigations are somewhat contradictory but if the most reliance is placed on the recent findings which have been obtained with improved techniques it must be concluded that for a single twitch little change in the ADP or ATP level can be demonstrated in either frog or turtle muscle. In organic phosphate does increase as a result of activity and from studies on the nucleotide and creatine phosphate levels after more prolonged activity it follows this phosphate has at some stage been derived from ATP. Whether the dephosphorylation of ATP is the immediate source of this phosphate or whether the precursor is some unidentified organic phosphate compound as is suggested by the recent work of Fleckstein *et al.* (42), is yet to be determined. Nevertheless the ATPase activity of the myofibril has a number of features which can be taken as circumstantial evidence of the probable function of this enzyme.

### *Myofibrillar ATPase*

The ATPase of the myofibril as observed *in vitro* may be merely a feature of the isolated system, for in the cell when the myofibril is closely associated with the sarcoplasm it is possible that the enzyme takes on some form of so far unrecognised transferring activity which ultimately involves the utilisation of the « energy rich » phosphate bond to bring about the shortening of the contractile elements. From this point of view it is of interest to draw an analogy with the muscle mitochondria. Like the mitochondria of liver these structures possess an ATPase which presumably plays an important part in their metabolic function although the normal role of this enzyme can only be surmized. For example the ATPase could play a part in oxidative phosphorylation, maintenance of osmotic relationships, active transport across the mitochondrial membrane, etc. If the mitochondrion is structurally intact this ATPase is not very active to added ATP, but once these structures have been damaged *i.e.* the internal environment is changed, the enzyme acts by splitting ATP at a high rate (44, 45).

A very important question is of course whether the myofibrillar ATPase is capable of liberating phosphate at a rate which is comparable with the maximum rate of phosphate production in activity. From the data of Lundsgaard and Meyerhof, Mommaerts (46) has concluded that at 20° C. the rate of inorganic phosphate production associated with tetanus in frog muscle is about $2 \times 10^{-4}$ mole/g. of muscle/min. He concludes on indirect and less conclusive grounds that during activity at 37° C. the rate of inorganic phosphate production in mammalian muscle is about $10^{-3}$ mole/g./min. This latter value is to some extent determined by the following assumptions : (1) that the resting rate of inorganic phosphate production at 37° C., calculated on the basis of oxygen consumption of dog gastrocnemius, is about 10 times as great as for the frog at 20 °C. (2) that the rate of inorganic phosphate production increases 500 times in activity, which seems a very liberal estimate although it is partly supported by oxygen consumption data. These figures for phosphate production correspond to the total amount produced by the muscle cell and although during activity the bulk must arise from the myofibril, part will also be produced by other metabolic processes and probably by the

electrical changes associated with stimulation. It may be concluded that in mammalian muscle the maximum rate of inorganic phosphate production by the contractile system is thus in the range of $1 - 10 \times 10^{-4}$ moles/g./min. The true value is probably nearer to the lower figure and to maintain such a rate of inorganic phosphate production at 37° C. the myofibril would have to split ATP at a rate which corresponds to a $Q_P$ value of approximately 1100, assuming that 1 g. of muscle contains 120 mg. of myofibrils. Isolated rabbit skeletal myofibrils in a medium of total ionic strength approximately 0.06, containing 0.005 M $MgCl_2$ and the same concentration of ATP at pH 7.4, have $Q_P$ values of 400-600 at 20° C., which correspond to values ranging from 1000-2000 at 37° C. Activities of this order are obtained with routine preparations and it is possible that *in situ* the enzyme is even more active for it is very difficult to avoid some oxidative inactivation or heavy metal contamination of the enzyme during purification. An important factor which must be considered is that Mg activation of the myofibrillar ATPase is rather sensitive to the ionic strength, for increasing the latter from 0.06 to 0.16 reduces the rate of ATP hydrolysis by a factor of approximately 2, and at higher ionic strengths Mg becomes inhibitory. The inhibitory action of Mg on myosin ATPase which is independent of the ionic strength and is antagonistic to Ca activation, has lead some workers (35, 36) to assume that it also applies in the cell and to conclude that the myosin ATPase would be functioning at such a low rate due to this effect that it could not account for the inorganic phosphate produced during activity. Such assumptions, however, are misleading for the presence of actin profoundly modifies the effect of Mg on the myosin ATPase. In the gel state, *i.e.* at low ionic strengths, this ion activates actomyosin ATPase and consequently in the presence of Mg the myofibril will hydrolyse ATP at almost the same rate as with Ca. If substrate is not in excess (figure 5) the addition of Ca to the myofibril system already activated by Mg has little effect on the rate of ATP splitting, but addition of Mg usually brings the activity obtained in the presence of Ca close to a rate which would be expected if Mg alone was present (figure 2).

It can be concluded that in the presence of either or both divalent cations under favourable ionic conditions the isolated myofibril can liberate inorganic phosphate from ATP at pH 7.4 at a rate which is about adequate to account for the speed of formation during activity in muscle.

The striking difference in behaviour of Mg and Ca in the myofibrillar ATPase system is to some extent parallelled by their effects on the glycerated fibre in the presence of the relaxing factor. With the aim of further understanding the role of these ions in the mechanism of the enzymic hydrolysis of ATP and their relation to the function of the enzyme in the muscle cell, their effects on certain features of the myofibrillar ATPase have been compared. Mg and Ca differ in two important ways in this system.

(*a*) Whereas the pattern of Ca activation is similar at 0° C and 20° C. and the $Q_{10}$ over this range is fairly normal for a hydrolytic enzyme, the activating effect

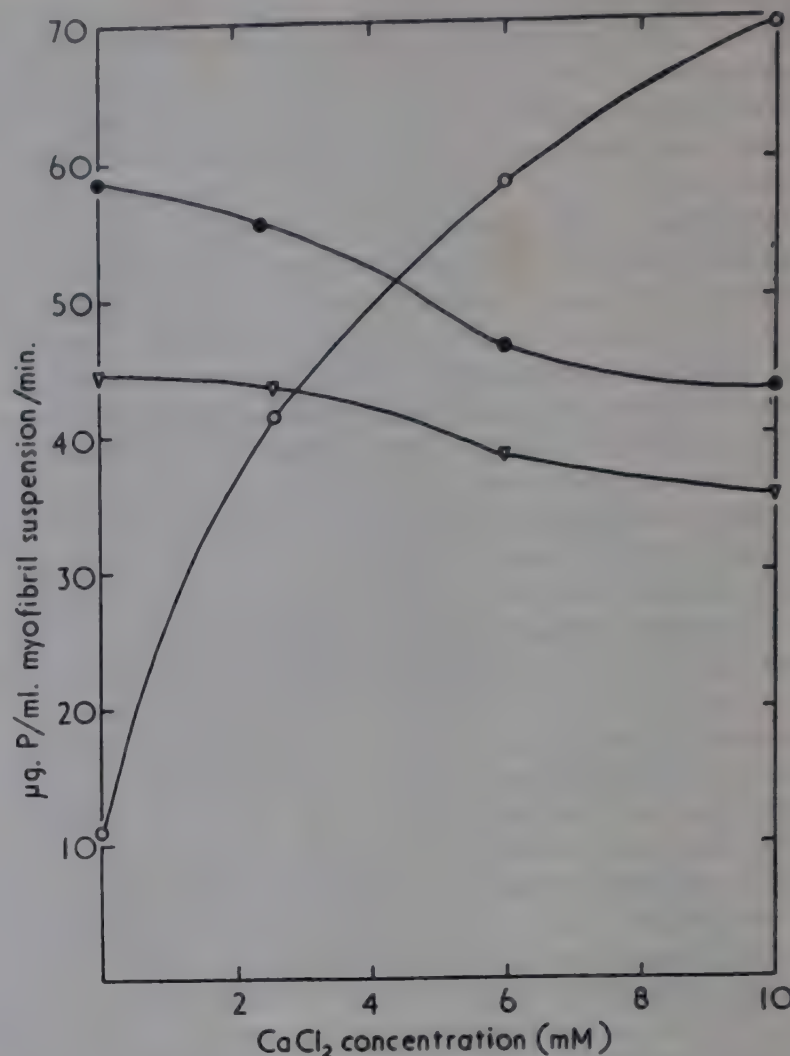


FIG. 2. — Effect of Mg on the Ca activated ATPase of rabbit skeletal myofibrils. All incubations carried out for 5 min. at 20° C. in medium which contained in addition to the activator, 0.005 M NaATP and 0.05 M Tris-HCl buffer pH 7.4.

○———○ No activator other than $CaCl_2$.
●———● 0.005 M $MgCl_2$ present throughout.
▽———▽ 0.010 M $MgCl_2$ present throughout.

of Mg on the myofibrillar ATPase is markedly altered by such a temperature change. At the lower temperature 0.001 M Mg inhibits and 0.005-0.01 M activates only very slightly; for example with 0.005 M $MgCl_2$ the rate of ATP hydrolysis at 0° C. is approximately 1/50 of the activity at 20° C. (47).

(*b*) When the myofibrillar ATPase is activated by Mg it is much more sensitive to inhibition by excess substrate than when Ca is the activating ion. In a system containing 0.005 M $CaCl_2$ the ATPase rate is practically unaffected by increasing the ATP concentration from $2 - 10 \times 10^{-3}$ M (figure 3), on the other hand when $MgCl_2$ is present instead of $CaCl_2$ if the molar concentration of ATP exceeds the concentration of $MgCl_2$, sharp inhibition sets in (figure 4).

The inhibitory effect of higher concentrations of ATP on the myofibrillar ATPase was reported by Hasselbach and Weber (48, 49) who showed that the relaxing factor lowered the level at which ATP became inhibitory when the assay conditions were otherwise kept constant. Further investigation of this effect has shown that there is a close relation between the Mg concentration and the concentration at which ATP becomes inhibitory to the myofibrillar ATPase. These findings have prompted

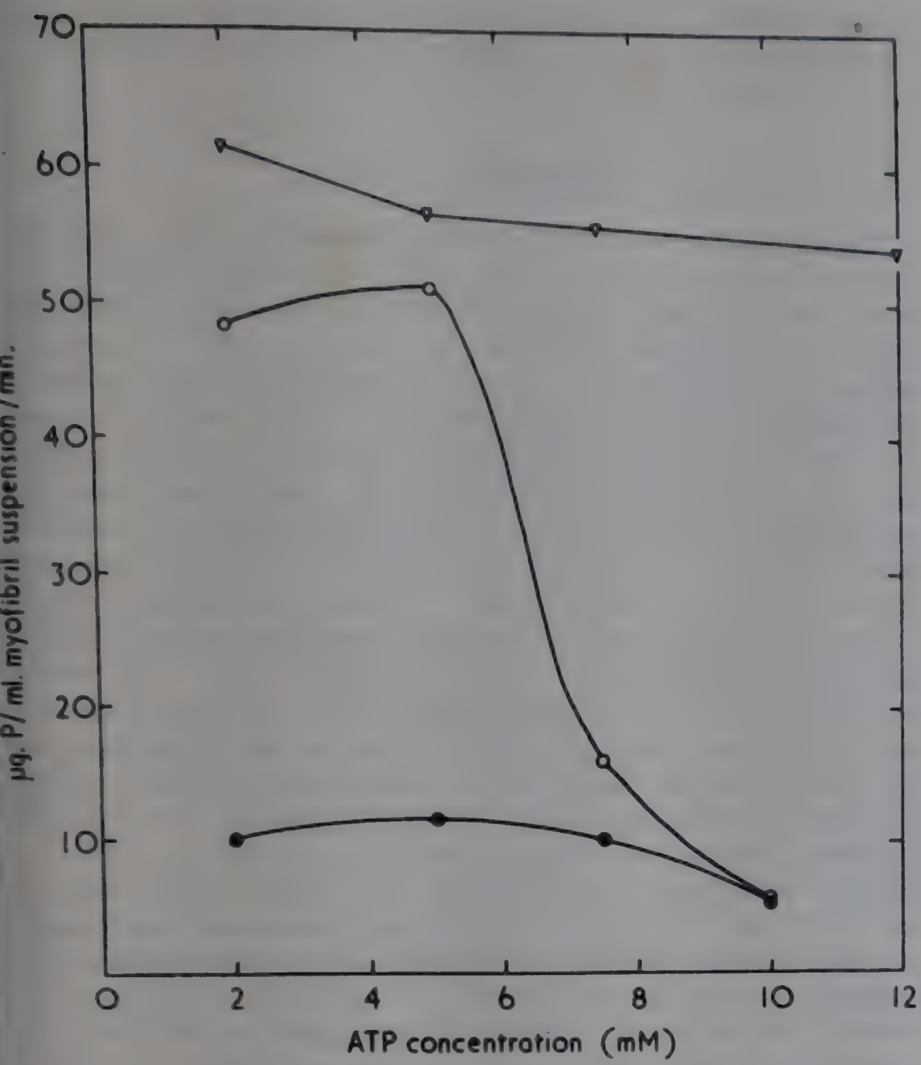


FIG. 3. — Effect of substrate concentration on the ATPase activity of rabbit skeletal myofibrils. Enzyme experiments carried out as for figure 2.

●———● No activator.
○———○ 0.005 M $MgCl_2$.
▽———▽ 0.005 M $CaCl_2$.

the suggestion (50) that the relaxing factor acts by binding Mg so that this ion becomes unavailable for the myofibrillar ATPase system (*). On this hypothesis in resting muscle most of the available Mg is bound to the relaxing factor and the ATP concentration is relatively much higher than that of the free Mg; consequently the myofibrillar ATPase is strongly inhibited. Stimulation of the muscle involves a momentary release of the Mg bound to the factor with the result that the ratio of Mg concentration to the concentration of ATP rises to a value near 1 and the rate of ATP hydrolysis increases sharply (figure 4). Relaxation sets in as soon as the active period ends for the Mg once more becomes bound to the factor and the ATPase falls again to the basal level. A feature of the theory is that it relies on changes in the free Mg level of the sarcoplasm whilst the concentration of ATP remains relatively unchanged as has recently been shown to be the case (42, 43). Quite a small change in Mg concentration *e.g.* 0.003 M, is sufficient to reduce the ATPase of isolated myofibrils to 1/3 to 1/5 of the original activity if the Mg and ATP concentrations are at first approximately equal. In whole skeletal muscle the total Mg and ATP content are in the right relative amounts, for of the two, the Mg is higher (51). A portion of the Mg will be bound to the relaxing factor to produce the inhibition of myofibrillar ATPase which occurs during relaxation and

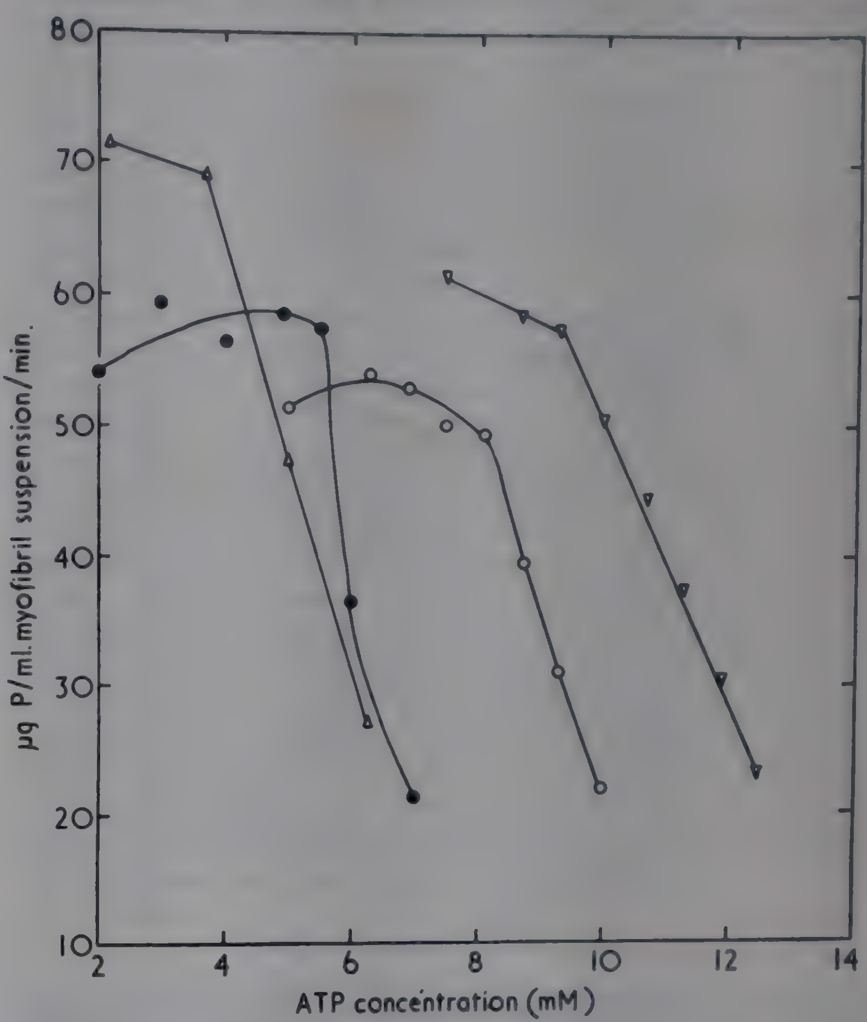


FIG. 4. — Relation between Mg concentration and substrate inhibition of rabbit skeletal myofibrillar ATPase. Enzyme experiments carried out as for figure 2, using different myofibril preparations for each set of points.

△———△ 0.0025 M $MgCl_2$.
●———● 0.005 M $MgCl_2$.
○———○ 0.0075 M $MgCl_2$.
▽———▽ 0.010 M $MgCl_2$.

this will be subsequently released on stimulation. Preliminary experiments suggest that the pyrophosphate group is essential for this inhibition as inorganic pyrophosphate also inhibits the Mg activated ATPase and this inhibition is likewise dependent on the Mg concentration. Adenylic and inosinic acids which possess no pyrophosphate group are not inhibitory when added to Mg activated myofibrillar ATPase systems. Clearly the evidence suggests that when magnesium is complexed, in approximately equimolar proportions, with the pyrophosphate group in ATP, or inorganic pyrophosphate, further addition of these pyrophosphate brings about inhibition of the ATPase compounds because these substances compete with the true substrate, a Mg-ATP complex, for the active centers of the enzyme. The ineffectiveness of the relaxing factor in the presence of Ca is readily explained for when activated by this ion

(*) Weber and Portzehl have suggested (86) a somewhat similar mechanism in that in resting muscle the relaxing factor binds Mg which is replaced by Ca during contraction.

the myofibrillar ATPase no longer shows inhibition the presence of excess ATP or ADP. Addition of Ca to systems containing Mg and ATP in the inhibitory ratio restores the ATPase to approximately its original value (figure 5). Likewise the relaxing effects of versene on glycerated fibres, which were obtained by Bozler (52) and which he explained on the basis of its ability to combine with Ca, are easier to understand if Mg is

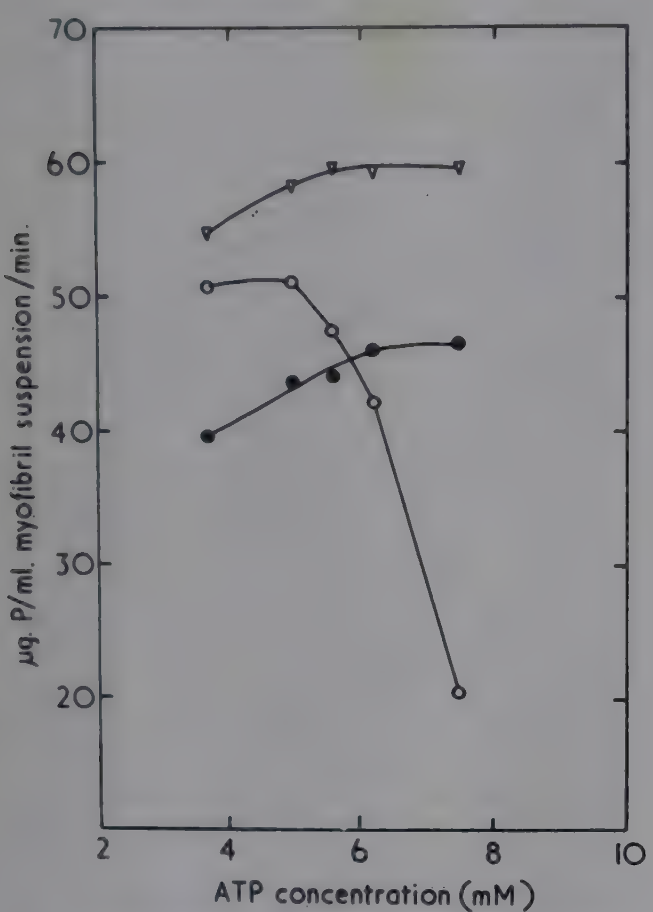


FIG. 5. — Comparison of the effect varying substrate concentration on Ca and Mg activated ATPase of rabbit skeletal myofibrils. Enzyme experiments carried out as for figure 2.
∇———∇ 0.005 M $CaCl_2$.
○———○ 0.005 M $MgCl_2$.
●———● 0.005 M $CaCl_2$ + 0.005 M $MgCl_2$

considered to be the activating ion which is chelated. It could well be that the relaxing action claimed for myokinase (53) and creatine phosphokinase (54) systems is due to competition for Mg between these enzyme systems and the myofibrillar ATPase.

The importance of Mg in the myosin ATPase system is suggested by the work of Friess *et al.* (55) as a result of further studies on the rather remarkable action of versene in stimulating the ATPase activity of myosin which has previously been treated with this chelating agent until it no longer activated by Ca (56). These workers find that magnesium is tightly bound to myosin and suggest that it acts as prosthetic group through which versene exerts its effect. Further indication that we have much to learn about the mechanism of myosin ATPase is the recent demonstration that the Ca activated ATPase of myosin (57) and myofibrils (47) can be stimulated by low concentrations of the sulphydryl poison, phenylmercuricacetate. Higher concentrations of this substance produced the inhibition of the ATPase which is usually reported with sulphydryl reagents. This observation is difficult to fit into the conventional ideas of myosin as enzyme requiring intact sulphydryl groups for activity.

### *Sarcoplasmic ATPase*

The myofibrillar ATPase is the main ATP splitting system in the muscle cell, but the sarcoplasm also possesses ATPase activity which is associated mainly with the granular components (44, 45, 58). Partially purified preparations of the sarcoplasmic ATPase have been obtained from rabbit and rat muscle (59) and there is evidence that in the rabbit at least the enzyme exists as a lipoprotein complex (60). No doubt this enzyme is similar in function to the mitochondrial ATPase of liver although its precise distribution in the sarcoplasm is not as yet entirely clear. Different types of muscle vary appreciably in the number and size of sarcosomes, present, the so-called I and A granules (61). The latter are typical of an active muscle such as pigeon breast and correspond closely in properties to the mitochondria of other tissues whereas in many mammalian white striated muscles, granules are much smaller and appear to be associated with the I band, as for example in the rabbit. Granular preparations from both these tissues have ATPase activity although the yields are very much smaller in the case of the rabbit. Medium speeds such as those used in the preparation of mitochondria sediment about 1/3-1/4 of the total ATPase activity from sarcoplasmic extracts and most of the remaining ATPase activity can be removed by further centrifugation under the conditions which normally bring down microsomes from extracts of tissues such as liver. This latter fraction as obtained from muscle probably contains the reticulated structures which Bennett and Porter (62) have observed in sarcoplasm and which correspond to the cytoplasmic reticulum present in liver and kidney cells. It is not easy however, at this stage, to decide whether the ATPase activity of the microsome fraction is an intrinsic property of these structures or whether it arises from small granules produced by the break-up of the larger sarcosomes.

Some ATPase activity remains in the supernatant after removal of mitochondria and microsomes from rabbit and pigeon breast sarcoplasm but it has yet to be determined whether this corresponds to a specific ATPase or to a pyrophosphatase such as has been reported in fly muscle (63).

The larger sarcosomes which are particularly rich in highly active tissues such as heart, and bird and insect flight muscles, apparently correspond to mitochondria. Pigeon breast mitochondria if isolated carefully show latent ATPase activity which can be activated by DNP or by procedures which disrupt their structure. The smaller granules found in rabbit skeletal muscle however, have much lower oxidative activity but probably do correspond to the mitochondria of other more active muscles as it can be demonstrated that they too possess latent ATPase activity when isolated in the Tris-KCl medium (45). A criterion of structurally intact liver mitochondria of high phosphorylating ability is low ATPase activity in isotonic media, and preparations which have barely detectable activity are readily obtained

from this tissue by differential centrifugation of homogenates made in 0.25 M sucrose. The best rabbit and pigeon breast granule preparations invariably have much higher basal ATPase activity when assayed in isotonic media compared with similar preparations from liver. This may be a real difference between liver and muscle mitochondria due perhaps to a difference in permeability to ATP, but it is possible that it could arise from the fact that Ca is liberated during homogenation of the tissues. This cation is found in comparatively large amounts in muscle and has deleterious effects on mitochondrial function in that it inhibits oxidative phosphorylation and activates the ATPase (64, 65). Such an explanation is not altogether satisfactory for even when isolated in media containing versene, freshly prepared pigeon breast and rabbit mitochondria still have appreciable ATPase activity in the absence of DNP. Mg, on the other hand, plays an important part in mitochondrial function as it is a cofactor in maintaining structural integrity as indicated by latency of the ATPase (45) and is essential for many of the metabolic activities of mitochondria in muscle and liver (66, 67). These facts taken with its role in activation of the myofibrillar ATPase and its importance in relaxation strongly support the view that Mg plays a unique role in cell metabolism in general and in muscle in particular. If extrapolation from the *in vitro* effects with intact intracellular structures is justified, there can be little free Ca present in sarcoplasm for this cation will disturb myofibrillar function by preventing relaxation and mitochondrial metabolism by activating the ATPase and uncoupling oxidative phosphorylation.

In addition to the somewhat analogous behaviour of Mg and Ca in the myofibrillar and mitochondrial systems the ATPases associated with these two structures possess many similar properties. To suggest that these similarities reflect fundamental similarities in function is perhaps taking the analogy too far, but certainly they indicate some identity in mechanism. The mitochondrial ATPase is in a way not yet understood, related to the phosphorylating function, but there is some evidence also that it is concerned in the reversal of swelling phenomenon (see below) which can be explained on the basis of the possession of a contractile system by the mitochondria. Certainly mitochondria as seen in living cells undergo marked changes in shape which imply the presence of such a system.

Some difficulties exist in making comparisons between the ATPase activity of intact myofibrils and mitochondria, for in the latter case the activity is readily stimulated many times by what appears to be purely mechanical effects, suggesting that structural and permeability factors may be important. In some cases studies of the mitochondrial ATPase have been carried out on aqueous extracts of acetone powders and these have been included wherever possible in table II. Much of the data on mitochondrial ATPase is derived from studies on preparations from liver but the evidence is that the muscle enzyme is fundamentally similar.

It is clear from table II that very marked similarities exist in the effects of various activators and inhibitors on the myofibrillar ATPase on the one hand and the widely distributed mitochondrial ATPases on the other. The effect of DNP on the former system is of particular

TABLE II

*Comparison of some properties of the myofibrillar and mitochondrial ATPases*

| Property | Myofibrillar ATPase | Mitochondrial ATPase |
|---|---|---|
| Specificity | ATP, ITP, UTP. | ATP, ITP, UTP ?<br>ADP very slowly attacked (68). |
| Activating cations | Ca (myosin).<br>Ca and Mg (myofibril). | Mg (rabbit muscle granules 58, 59).<br>Mg and to lesser extent Ca (acetone powder rat liver mitochondria and aged whole mitochondria from rat liver and pigeon breast muscle, 44). |
| Mg activation at 0° C. | Absent. | Absent (rat liver mitochondria acetone powder). Little or no activation (whole fresh rat liver and pigeon breast mitochondria, even in presence of 0.1 mM DNP). |
| Sulphydryl reagents | Inhibition at higher concentrations.<br>Low concentrations of PMA stimulates Ca activation of myosir (57) and myofibrils (47). | Inhibition at higher concentrations.<br>Aged rat liver mitochondria ATPase stimulated by *o*-iodosobenzoate (69). PMA inhibits pigeon breast mitochondrial ATPase (70). |
| DNP | $10^{-3}$ M stimulates Ca activation of myosin and actomyosin, and myofibrils at higher ionic strengths (47, 57).<br>Effect reversible.<br>ITPase of myosin not stimulated (57). | $10^{-4}$ M stimulates Mg activation of rat liver mitochondrial powder and to much greater extent activity of whole rat liver and pigeon breast mitochondria.<br>Effect reversible.<br>ITPase of pigeon breast mitochondria not stimulated (70). |
| Sulphydryl reagents on DNP stimulation | Low concentrations of PMA, themselves stimulatory, abolish DNP stimulation of myosin ATPase (57). | PMA abolishes DNP effect on pigeon breast mitochondria (70).<br>*p*-chloromercuribenzoate abolishes DNP effect on rat liver mitochondria (69). |

interest for it is another example of how the enzymic activity of myosin is modified by the presence of actin which itself has no enzymic activity in the conventional sense although there is evidence that the polymerisation of G to F-actin involves the conversion of ATP to ADP (71, 72, 73). At low ionic strength the addition of increasing amounts of actin to myosin reduces the stimulatory effect of DNP on the ATPase until when the ratio is approximately 3 parts of myosin to 1 of actin the effect is almost abolished. In general on increasing the ionic strength differences in behaviour between myosin and actomyosin or myofibrils disappear. On the basis of the uncoupling action of DNP on mitochondrial phosphorylation one wonders whether there is anything analogous in its action on the myosin ATPase, perhaps for example a « short-circuiting » of the mechanism of phosphorylation of myosin itself which becomes evident as an increase in the rate of ATP hydrolysis. From this viewpoint, however, it should be mentioned that the presence of DNP does not cause any obvious modification of the ATP induced contraction of isolated myofibrils. Whatever the mechanism of DNP stimulation, the effect disappears in the presence of actin and it can only be supposed that the protein prevents the DNP from exerting its action at the enzymically active centres of myosin either by associating with them itself or by complexing with the DNP.

ATP can also bring about structural changes in mitochondria. For example freshly prepared pigeon breast mitochondria swell up if transferred to hypotonic media, but if this process is not allowed to progress too far it can be reversed by the addition of low concentrations of ATP (45). This reversal of swelling can be conveniently followed by measuring the changes in optical density at 520 mμ of mitochondrial suspensions (figure 6) and the validity of this method was confirmed by direct estimations which indicated that up to 50 % of the total water in swollen pigeon mitochondria was lost on application of ATP. The only substances whose application at very low concentrations has so far shown to bring about the reversal of swelling are ITP and ATP. Somewhat similar effects can be obtained with hypertonic salt solutions but the ATP effect cannot be explained on the basis that the nucleotides bring about the reversal of swelling by causing similar changes in tonicity, for the nucleotide effect is extremely specific and the concentrations required to produce it are of a different order of magnitude. Another significant feature is that the ATP effect is only obtained when the mitochondria possess latent ATPase activity. DNP in concentrations which activate the mitochondrial ATPase destroy the reversal of swelling effect.

One possible explanation of this ATP effect which has already been mentioned, is that the mitochondrial system contains a contractile protein similar to actomyosin in that it requires ATP to function normally. If such a contractile system formed part of the mitochondrial membrane or even an integral part of the whole structure, contraction would involve a decrease in volume with a squeezing out of water, a kind of synaeresis. The analogy is given a certain degree of substance by the fact that the hydrolysis of ATP is in some way related to the reversal of swelling, although in view of the DNP effect

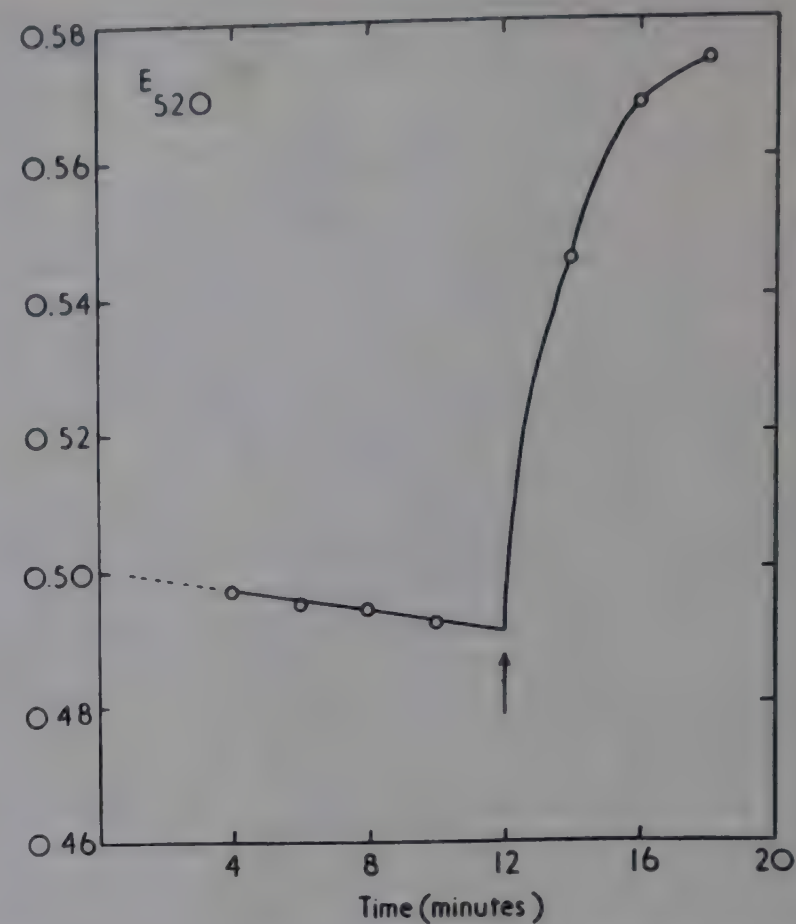


Fig. 6. — Reversal of swelling of pigeon breast muscle mitochondria. The effect of ATP on the optical density of a mitochondrial suspension. 0.1 ml. of mitochondrial suspension was added at zero line to a medium containing 0.1 M KCl, 0.005 M $MgSO_4$ and 0.05 M Tris-buffer, pH 7.4. Total volume 3 ml.; temperature 18° C. After 12 min., 5 μ-moles ATP added (*arrow*).

this is not a simple relation. Nevertheless the rate of reversal can be correlated to the rate of ATP hydrolysis in so far as the latter can be made to vary by changes in substrate concentration and pH in various buffer systems (70).

### *Nucleotide content*

Recent analyses of the nucleotide content of resting skeletal muscle indicate that a considerable amount of the adenine nucleotide is present as ADP. As judged by the level of oxygen consumption (46) the resting rate of inorganic phosphate production is low, nevertheless appreciable quantities of inorganic phosphate are present in resting muscle (42). It might be expected that if the ADP and the excess inorganic phosphate are both freely available to the glycolytic and oxidative systems then this nucleotide would exist in the fully phosphorylated form. Certainly with both aerobic and anaerobic phosphorylating systems (74) *in vitro*, so long as there is sufficient acceptor, inorganic phosphate is taken up efficiently. The possibility exists, however, that some of the ADP is unavailable to the normal phosphorylating systems of muscle. For example freshly isolated rabbit skeletal myofibrils contain ADP tightly bound to the structure so that it is unavailable to the sarcoplasmic enzymes which rapidly convert any free ADP to inosinic acid in homogenates (75). The ADP present in the washed isolated myofibrils is stable to quite prolonged storage at 0° C. and probably is associated with the actin component. The possibility exists that this ADP

is converted to ATP which acts as a 'built in' source of energy rich phosphate, but in so far as it is unavailable to the creatine phosphokinase and myokinase systems there is no evidence as yet to suggest that the myofibrillar bound ADP can be phosphorylated *in situ*. In this respect it differs from the small amounts of nucleotide found in the mitochondria of pigeon breast and granules of rabbit skeletal muscle which, when fresh, contain nucleotides mainly as ATP and ADP. The degree of phosphorylation of mitochondrial adenine nucleotides is very dependent on the metabolic state of these structures (76), and when preparations from muscle are stored at 0° C. for a few days the nucleotides break down to inosinic acid and inosine under the influence of the enzymes present (77).

ATP and ADP make up the bulk of the nucleoside polyphosphates and apart from about 10 % of the total which is bound to the myofibrillar structure and an unknown amount, probably quite small, associated with the mitochondria, the remainder is presumably in free solution in the sarcoplasm. Recently chromatographic evidence for the presence of other nucleotides has been obtained. Bergkvist and Deutsch (78) has demonstrated that GTP and UTP are both present in amounts of 1-2%. ITP is also found in small amounts in ATP samples prepared from rabbit muscle and presumably this arises from IDP by the action of nucleoside diphosphokinase (79, 80) :

$$ATP + IDP \rightleftarrows ITP + ADP$$

The IDP required for this reaction may be produced by the action of ADP deaminase which was demonstrated by Webster (81) and Deutsch and Nilsson (82) to be present in rabbit skeletal muscle. This enzyme is distinct from 5-adenylic acid deaminase because purified myosin preparations which have 5-adenylic acid deaminase activity do not deaminate ADP; purified myofibril preparations at pH 6.1 possess ADP deaminase activity although it is only a few % of the activity to adenylic acid (77).

The study of muscle chemistry has been characterised by successive waves of interest each focussed sharply on some substance considered at the time to be of particular significance for the contractile process. ATP has held this position unassailed for a number of years although evidence is now available which makes it imperative to reexamine its role in contraction. Certainly inorganic phosphate is produced during a single twitch but although the level of ATP and ADP remain unchanged an unknown organic phosphate compound disappears in amounts which would account for the increase in inorganic phosphate (42). If the direct source of inorganic phosphate is ATP then rephosphorylation must take place instantly and neither of the recent investigations (42, 43) have eliminated the possibility that this is accomplished by anaerobic glycolysis. The alternative explanation is that some other substance is the direct source of the inorganic phosphate produced on contraction. It is possible that *in situ* one of the other nucleoside triphosphates is the preferred substrate, for although these substances occur in relatively small amounts they would probably be kept fully phosphorylated by the nucleoside diphosphokinases at the expense of the ATP which is present in much higher concentration. Of the nucleoside triphosphates so far tested there is evidence that UTP is split considerably faster than ATP (83). Nevertheless it is difficult to imagine that *in situ* the myofibril does not break down directly appreciable amounts of ATP. For unless the ATP is in some way rendered unavailable to the myofibril the very much higher concentrations of the latter would offset a considerable disparity of affinity which might exist between the myofibrillar ATPase and ATP on the one hand and some other nucleoside triphosphate on the other.

Irrespective of the course of events *in situ* ATP will bring about the contraction of the isolated myofibril washed free from all soluble cofactors and there is good evidence that contraction in this system will only take place whilst the ATP is being dephosphorylated at an appreciable rate (84). In view of these effects, its importance in the motility of many other biological systems (85) and the physical changes induced by this substance in purified actomyosin systems there is convincing evidence of the direct implication of ATP wherever reversible structural changes occur in living material.

*Acknowledgements*

The author wishes to express his thanks for the expert technical assistance of Mr. T. C. Grey in a number of the unpublished experiments described in this communication, and for the continued support of the Medical Research Council in the form of a Research Expenses Grant.

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## Addendum

(*Received* 15 *September* 1955)

Since this manuscript was submitted further work in collaboration with Dr. Corsi has strongly suggested that the myofibrillar pseudoglobulin extracted with tropomyosin is derived from actin. This conclusion has been reached, despite the electrophoretic data, presented in figure 1, for a protein apparently identical in properties to the pseudoglobulin has been obtained from F-actin by dialysis against 0.078 M borate buffer pH 7.1. Even after prolonged extraction the pseudoglobulin obtained from the myofibril represented only part of the total actin present. This suggests that in the myofibril actin is present in two different states of association, only one of which can be readily converted to the inactive soluble form by treatment with borate. It is possible that the latter extractable form of actin is confined to the I band whereas the actin of the A band which is associated with myosin is not readily converted to the pseudoglobulin.

# La biochimie du cerveau

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(*Reçu le* 21 *avril* 1955)

Le problème de la biochimie du cerveau, des fondements chimiques de son activité, est un des problèmes les plus intéressants et en même temps un des plus difficiles de la chimie biologique. La diversité des structures cellulaires et conductrices, la distribution extrêmement compliquée de la substance grise et de la substance blanche et la richesse du tissu cérébral en corps labiles rendent l'étude de la composition chimique du cerveau et de son métabolisme très difficile.

A l'heure actuelle, nous disposons de beaucoup de données expérimentales dans le domaine de la statique et de la dynamique biochimiques, et de la biochimie fonctionnelle du cerveau qui nous permettent dans une certaine mesure de comprendre les particularités de l'activité nerveuse.

Je voudrais exposer dans ce rapport les résultats des recherches dans le domaine de la biochimie du cerveau poursuivies depuis plusieurs années à l'Institut de Biochimie de l'Académie des Sciences de la République Socialiste Soviétique d'Ukraine, de même que les résultats des travaux récents d'autres savants.

Le principe fondamental de la physiologie moderne est la conception de l'intégrité et de la plasticité de l'organisme animal lié inséparablement aux modifications du milieu qui exercent une influence sur son état, sur le développement et le changement de ses propriétés fonctionnelles et de ses formes dans l'ontogénèse et dans la phylogénèse.

Chez les animaux supérieurs, chez l'homme en particulier, l'intégrité de l'organisme et ses relations avec le milieu intérieur et extérieur sont réglées et organisées, ainsi que l'ont indiqué très clairement les recherches de Setchénov et Pavlov, par le système nerveux central qui coordonne toutes les fonctions de l'organisme animal.

La biochimie moderne des animaux et de l'homme, qui doit être une biochimie fonctionnelle, a pour but principal l'étude du métabolisme de l'organisme entier dans les différents états fonctionnels et sous l'influence du milieu extérieur et l'éclaircissement des interrelations entre les fonctions spécifiques des organes et des systèmes et les particularités de leur métabolisme, avant tout du système nerveux, aussi bien que l'éclaircissement du mécanisme de la régulation des processus métaboliques par le système nerveux.

Dans nos recherches portant sur la biochimie du cerveau nous avons cherché à établir s'il existe des différences de composition chimique et des variations du métabolisme entre les parties du cerveau remplissant des fonctions différentes et si les processus du métabolisme des différentes parties du cerveau se modifient sous l'influence des divers facteurs.

### *Les méthodes des recherches*

Les rapports entre modifications fonctionnelles et métabolisme du cerveau ont été étudiés au cours d'expériences *in vivo* et *in vitro*. Les expériences sur les coupes histologiques et sur le tissu cérébral homogénéisé ont une valeur purement auxiliaire puisque les expériences *in vitro* ne peuvent que mettre en évidence le mécanisme des réactions métaboliques, tandis que les expériences *in vivo* peuvent révéler leur signification physiologique et leur rôle fonctionnel.

La préparation de coupes de tissu cérébral possédant un métabolisme rapide et une sensibilité exceptionnelle aux influences physiologiques peut provoquer la dégradation de plusieurs substances très importantes pour le fonctionnement du cerveau, par exemple, de la phosphocréatine, de l'ATP, de la cozymase. De plus, l'excitation des éléments nerveux dépourvus des connexions naturelles avec d'autres parties du cerveau peut provoquer des changements qui diffèrent de ceux qui se développent dans le cerveau intact. Dans le cerveau intact les phénomènes peuvent se dérouler autrement que dans les coupes, ce qui a été confirmé par les résultats de recherches qui ont montré que le même agent peut provoquer *in vitro* un effet entièrement différent, ou même opposé à celui qu'il provoque *in vivo*. Ainsi, par exemple, sous l'influence de certains composés barbituriques, la quantité de phosphocréatine dans le cerveau augmente *in vivo* (1, 2), tandis qu'elle baisse *in vitro* (3) ; les variations de la quantité de phosphate inorganique se font en sens inverse. Parfois les résultats des expériences *in vivo* sont identiques aux résultats des expériences sur les coupes. Ainsi, par exemple, les narcotiques inhibent la respiration des coupes du tissu cérébral (4) aussi bien que celle du cerveau intact (5) de l'homme et des animaux. C'est pourquoi, pour obtenir les données préliminaires, on peut, compte tenu des erreurs possibles, étudier les problèmes de la biochimie fonctionnelle du cerveau, en se servant des résultats des expériences *in vitro*.

D'autre part, quoique les expériences *in vivo* sur les animaux entiers soient très difficiles au point de vue technique, elles seules peuvent nous renseigner d'une manière définitive sur les interrelations entre les modifications fonctionnelles et les processus métaboliques du cerveau.

Dans les recherches sur la composition chimique du cerveau et son métabolisme (en particulier le métabolisme des composés phosphorés labiles) la méthode utilisée pour sacrifier les animaux joue un très grand rôle, de même que la méthode d'interruption des processus du métabolisme dans le cerveau.

Kerr (6) a appliqué la congélation du cerveau par l'air liquide : il anesthésiait les animaux puis chez les grands animaux, les soumettant à la respiration artificielle, il ouvrait la boîte crânienne et versait de l'air liquide sur les hémisphères (chez les petits animaux l'ouverture de la boîte crânienne ne fut pas effectuée), après quoi il retirait le cerveau. Cependant, il faut tenir compte qu'ici l'anesthésie peut à elle seule provoquer des altérations du métabolisme.

Pour éviter l'influence de l'anesthésie on peut (chez les grands animaux) décapiter l'animal promptement, retirer les hémisphères et les congeler par l'air liquide. Ou bien on peut plonger la tête (des petits animaux) détachée dans l'air liquide et retirer le cerveau après la congélation. La décapitation peut être la cause d'une forte irritation du cerveau et d'une dégradation des composés labiles. Pour éviter cet effet, on peut avant la décapitation injecter de l'hexenal (1 mg. d'une solution à 10 %). Les recherches de Ferdman et Dvornikova (7) font ressortir que les résultats du dosage de l'ATP et de la phosphocréatine dépendent de la technique employée dans l'expérimentation sur le cerveau.

On peut aussi (8,9) plonger le petit animal (rats, souris) tout entier dans l'air liquide. Ce procédé provoque sans doute une excitation, quoique peu prolongée, des analyseurs cutanés, qui a le temps d'agir sur le cerveau avant qu'il soit congelé ; elle fournit toutefois les chiffres les plus élevés pour l'ATP et la phosphocréatine.

Depuis les vingt dernières années on utilise les radioisotopes pour l'étude du métabolisme du tissu nerveux. Grâce à eux il a été possible d'établir que plusieurs composés que l'on considérait comme incapable de subir des transformations dans le cerveau de l'animal adulte, en réalité se décomposent et se synthétisent continuellement.

Pour l'étude des processus du métabolisme dans le cerveau on emploie surtout le phosphore marqué ($^{32}P$) parce que les composés phosphorés jouent le rôle le plus important dans le métabolisme de l'énergie et parce que les composés phosphorés sont les premiers à subir des transformations.

### *Protéines*

Les protéines jouent un rôle très important dans le fonctionnement du système nerveux central, comme Danilevsky (10) l'a signalé en 1891.

Toutefois l'étude des protéines du cerveau a peu progressé depuis les recherches d'Ewald (11), de Halliburton (12) et de Danilevski.

Il est difficile d'étudier les protéines cérébrales parce que le cerveau est très riche en lipides et que chaque tentative de séparer les lipides des protéines à l'aide de solvants organiques entraîne la dénaturation des protéines.

D'après les recherches de plusieurs auteurs (13) la majeure partie des protéines se trouve dans l'écorce des grands hémisphères du cerveau ; il y en a moins dans la substance blanche du cerveau ; encore moins dans la moelle épinière ; les nerfs périphériques en ont la plus petite quantité. Notre étude de diverses parties de la matière grise a montré que la plus grande quantité des protéines se trouve dans la partie de la substance grise qui est la plus jeune au point du vue phylogénétique et la plus compliquée au point de vue de la fonction, c'est-à-dire dans la substance grise des grands hémisphères du cerveau ; il y en a moins dans la substance grise de l'écorce du cervelet et des ganglions sous-corticaux ; il y en a encore moins dans la substance grise de la moelle épinière.

La substance grise et la substance blanche du cerveau diffèrent, selon les recherches de Palladine (15) non seulement par la quantité totale de leurs protéines, mais aussi par la qualité des substances protéiqnes, c'est-à-dire par la proportion de différentes fractions protéiques : la substance grise contient plus de protéines solubles dans l'eau et moins de résidu insoluble que la substance blanche.

On a commencé dernièrement à utiliser pour l'étude du métabolisme des protéines la méthode des isotopes.

Friedberg, Tarver et Greenberg (16), en étudiant l'incorporation de la méthionine marquée par le radiosoufre ($^{35}S$) dans les protéines de divers organes du rat, ont constaté qu'après l'injection intraveineuse de méthionine, l'intensité de l'incorporation du $^{35}S$, en d'autres termes la vitesse de renouvellement des protéines, est beaucoup plus faible dans le cerveau que dans d'autres organes. Mais si la méthionine est injectée dans les ventricules (au-delà de la barrière hématoencéphalique), l'incorporation dans le cerveau est plus intense que dans d'autres organes et tissus. Ces auteurs ont également constaté que la sortie de la méthionine marquée des protéines de divers tissus correspond bien à son incorporation ; ils pensent qu'il est préférable de mesurer la vitesse de renouvellement des protéines d'après la courbe de leur sortie.

Gaitonde et Richter (17) admettent que de la relation entre l'activité spécifique du soufre des protéines et celle du soufre soluble dans les acides aminés on peut caractériser la vitesse de la synthèse de protéine, et ont conclu que la vitesse de la synthèse des protéines dans le cerveau est comparativement grande (plus grande, par exemple, que dans le foie).

Ayant voulu établir la vitesse du renouvellement des protéines dans les diverses parties du système nerveux central, Palladine et Vertaimer (18) ont étudié l'intensité de l'incorporation de la méthionine marquée par $^{35}S$ dans les protéines de diverses sections du cerveau du chat. Nos recherches ont montré que la vitesse de renouvellement des protéines est la plus grande dans l'écorce des hémisphères et dans le cervelet, c'est-à-dire dans les

parties phylogénétiquement les plus jeunes et fonctionnellement les plus compliquées. La plus petite vitesse de renouvellement est mesurée dans la moelle épinière, c'est-à-dire la partie phylogénétiquement la plus ancienne et fonctionnellement la moins compliquée. L'intensité de renouvellement des protéines dans la matière blanche des grands hémisphères est voisine de celle de la moelle épinière. D'autres parties occupent une position intermédiaire (figure 1).

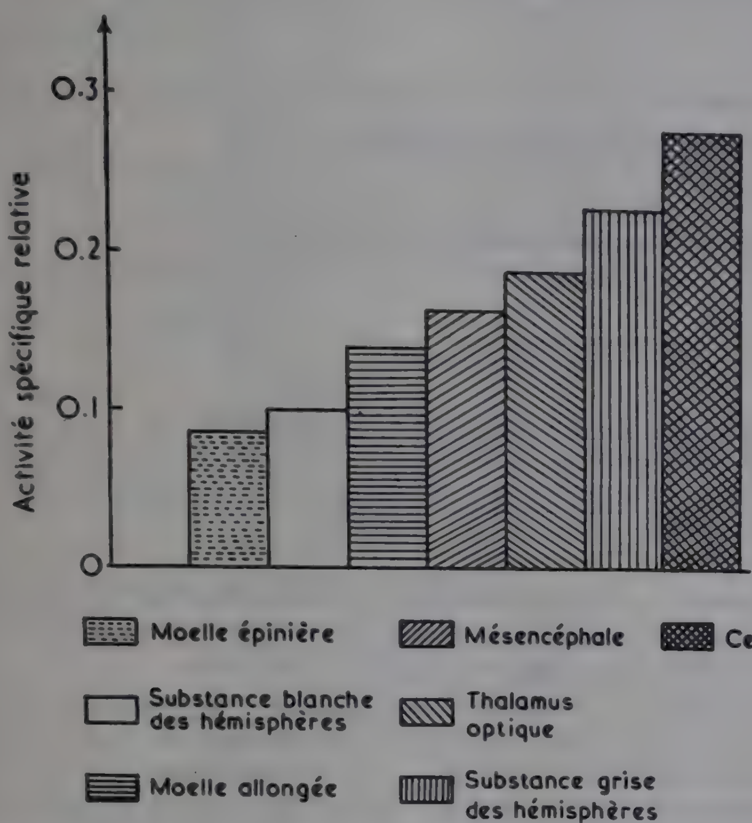


FIG. 1. — Activité relative spécifique des protéines des diverses parties du système nerveux central du chat.

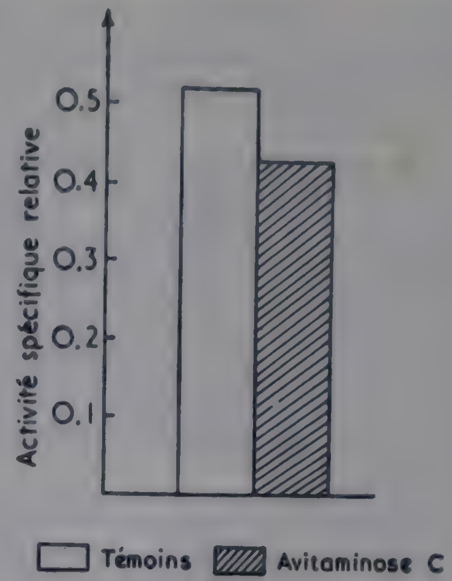


FIG. 2. — Activité relative spécifique des protéines du cerveau de cobaye en avitaminose C.

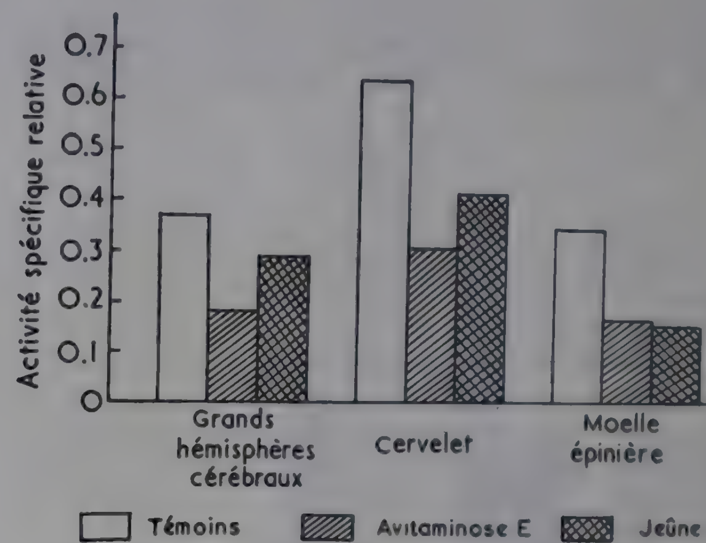


FIG. 3. — Activité relative spécifique des protéines du système nerveux central du lapin en avitaminose E ou au cours du jeûne.

Nos résultats confirment les observations de Cohn, Gaitonde et Richter (19) qui, en étudiant à l'aide de la technique radioautographique l'incorporation du $^{35}S$ dans les protéines du cerveau après les injections intracisternales ou intrapéritonéales de la méthionine marquée, ont constaté que l'incorporation est plus intense dans la substance grise que dans la matière blanche.

Si on n'a pas encore réussi à déterminer d'une façon précise la vitesse absolue de renouvellement des protéines du cerveau, la vitesse relative de renouvellement des protéines dans diverses parties du cerveau est déjà élucidée dans une certaine mesure.

Nous avons aussi étudié (18) le renouvellement des protéines du cerveau au cours des avitaminoses C et E. Au cours de l'avitaminose C on peut constater une légère diminution de l'intensité de renouvellement des protéines du cerveau des cobayes (figure 2). L'avitaminose E exerce une influence plus marquée sur le métabolisme protéique du cerveau : la vitesse de renouvellement des protéines dans les grands hémisphères du cerveau, dans le cervelet et dans la moelle épinière chez les lapins carencés en vitamines E tombe en moyenne à 50 %. Au cours du jeûne seul, la baisse de l'intensité de renouvellement des protéines dans les grands hémisphères et dans le cervelet du lapin est beaucoup moins accusée (figure 3).

Les nucléoprotéides (ribonucléoprotéides et désoxyribonucléoprotéides) jouent un rôle primordial dans le cerveau.

Hyden (20), étudiant à l'aide d'une technique roentgenmicroradiographique les coupes congelées des cellules nerveuses, a réussi à obtenir des données très intéressantes. Déterminant le taux des lipides, des pentose-nucléoprotéides et des protéines, il a établi que la constitution chimique des cellules nerveuses change avec l'âge : le taux des nucléoprotéides diminue, aux dépens des lipides qui les remplacent.

Au cours de l'intense activité du neurone (pendant la stimulation adéquate) le taux de nucléoprotéides baisse, tandis que la quantité des protéines ne change pas. Ainsi, il existe deux types de changements chimiques dans la cellule nerveuse : les uns dépendent de l'âge et se déroulent lentement ; les autres, qui sont permanents, consistent en dégradation et resynthèse des nucléoprotéides ; on peut penser qu'ils constituent la base chimique de l'activité du neurone.

Boulankine et ses collaborateurs (21), qui ont étudié les modifications des protéines structurelles du cerveau des rats blancs, ont constaté que les composés du type

lipoïde remplacent avec l'âge les acides nucléiques. Des changements similaires se produisent dans l'ensemble du tissu cérébral. En même temps, les nucléoprotéines s'enrichissent en protéines.

Les données de la littérature indiquent qu'il existe dans les tissus un certain rapport entre l'intensité de la synthèse des protéines et la teneur en acides nucléiques. La présence d'une quantité considérable d'acides nucléiques dans le tissu nerveux permet de supposer qu'ils jouent un rôle important dans l'activité du système nerveux; aussi l'étude du métabolisme des acides nucléiques du tissu cérébral et de leur participation dans l'activité physiologique du cerveau doit être l'objet d'une étude toute particulière.

Skvirskaja et Silitch (22) ont constaté que l'écorce du cerveau et la substance blanche sont très semblables l'une à l'autre, en ce qui concerne la teneur totale en acides nucléiques et la teneur des fractions isolées, qui dominent dans une certaine mesure dans le cerveau.

La teneur en acides nucléiques et en particulier en acide désoxyribonucléique est plus élevée dans le cervelet que dans la substance grise, et surtout plus élevée que dans la substance blanche des grands hémisphères (figure 4).

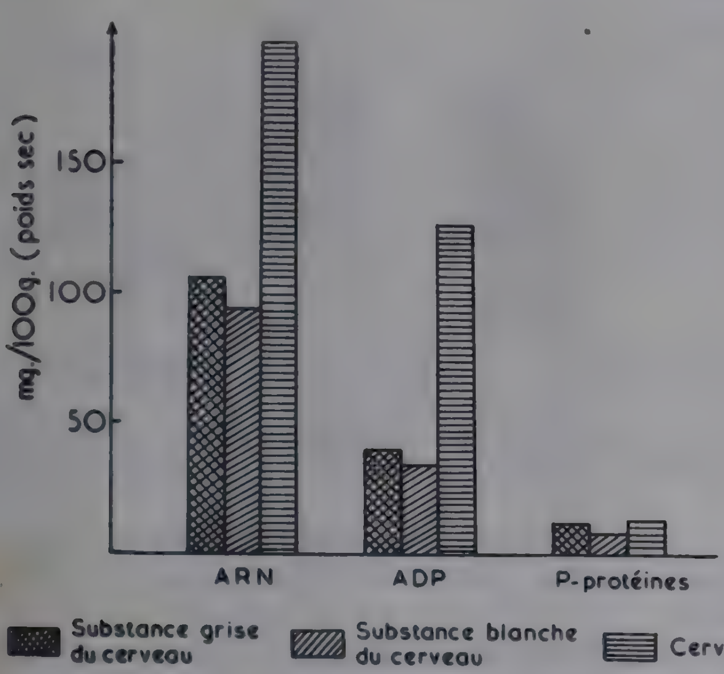


FIG. 4. — Teneur en acides nucléiques et en phosphoprotéines du cerveau et du cervelet de lapin.

A l'aide de la méthode de Palladine, Raschba et Schtutman (23) qui permet de séparer les noyaux de la substance grise du cerveau et d'établir que le nucléoprotéide des noyaux est le désoxyribonucléoprotéide et que l'acide ribonucléique (ARN) représente 20-30 % des acides nucléiques totaux (70-80 % étant présents sous forme d'acide désoxyribonucléique, ADN), on a constaté (22) que dans les noyaux du cervelet l'ARN représente seulement 12-13 % et l'ADN 87-88 % des acides nucléiques totaux ; ainsi les noyaux du cervelet contiennent moins d'acide ribonucléique que les noyaux de l'écorce des grands hémisphères. Ces observations nous font croire qu'il existe un certain rapport entre la composition des noyaux cellulaires et la fonction des cellules.

L'activité de la ribonucléase et de la désoxyribonucléase est plus élevée dans la substance grise et moins élevée dans la substance blanche du cerveau ; le cervelet occupe une place intermédiaire comme on peut s'en rendre compte à la lecture du tableau I et de la figure 5. Ce fait

TABLEAU I

*Activité de la ribonucléase (RNase) et de la désoxyribonucléase (DNase) du cerveau et du cervelet*

| | RNase | DNase |
|---|---|---|
| Substance grise des grands hémisphères du cerveau . . . . . . . . . . . . . . . | 7.92 | 1.40 |
| Substance blanche des grands hémisphères du cerveau . . . . . . . . . . . . . | 3.03 | 1.00 |
| Cervelet . . . . . . . . . . . . . . . . | 4.14 | 0.85 |

L'activité de la ribonucléase est exprimée en mg.P/mg.N libérés en une heure d'incubation; l'activité de la désoxyribonucléase est représentée par la différence entre la viscosité relative initiale et finale.

nous permet de supposer que le métabolisme des acides nucléiques est plus intense dans la substance grise que dans la substance blanche (22).

Ces derniers temps on a porté une attention toute particulière aux méthodes de détermination des acides nucléiques dans le cerveau, car le fractionnement des ribonucléoprotéides par la méthode de Schmidt et Thannhauser fournit des fractions contaminées par d'autres substances (24, 25, 26).

L'analyse du renouvellement du phosphore des acides nucléiques du cerveau intact (22, 27, 28), de même que dans les coupes du cerveau (29) à l'aide de radiophosphore ont montré que le phosphore de l'acide

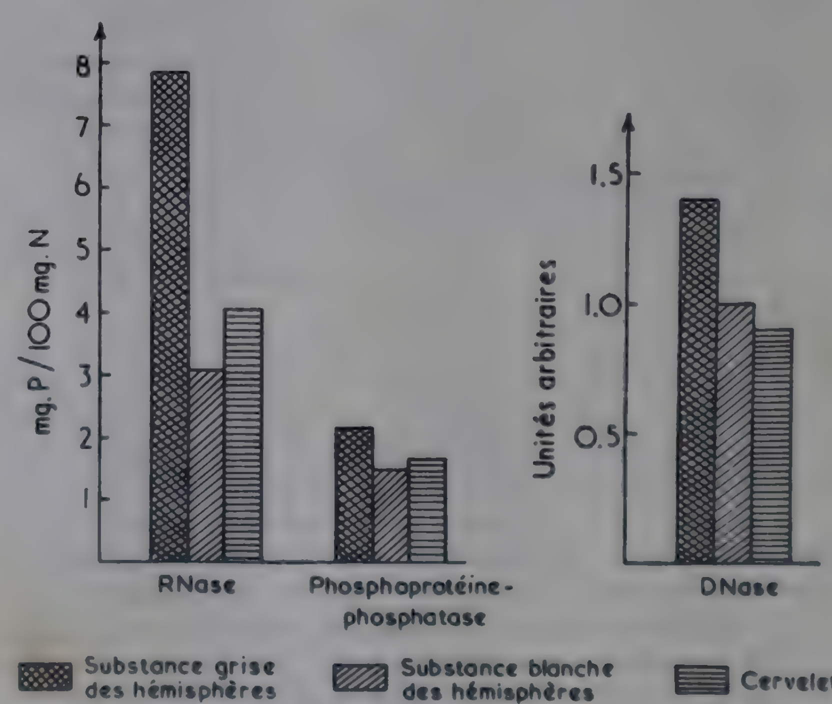


FIG. 5. — Activité de la ribonucléase (RNase), de la phosphoprotéine-phosphatase et de la désoxyribonucléase (DNase) du cerveau et du cervelet de lapin.

désoxyribonucléique s'échange très lentement, celui de l'acide ribonucléique un peu plus vite, mais toutefois plus lentement que celui des phosphoprotéines. En étudiant le métabolisme du phosphore des acides nucléiques dans diverses parties du cerveau, nous avons (22) établi que la vitesse de renouvellement du phosphore de l'acide ribonucléique dans la substance grise du cerveau des lapins est inférieure à celle de la substance blanche et du cervelet.

Des données analogues ont été obtenues par Kreps (28). Il a constaté que la vitesse de renouvellement de l'acide ribonucléique de l'écorce du cerveau de lapin était inférieure à la vitesse dans le cervelet, le cerveau intermédiaire et le cerveau moyen et allongé de l'animal ; on a constaté que chez les chiens la vitesse de renouvellement de l'acide ribonucléique est plus élevée dans les grands hémisphères que dans les autres sections, parce que les grands hémisphères du chien sont plus développés fonctionnellement.

La grande vitesse de renouvellement de la matière blanche du cerveau témoigne que la substance blanche n'est pas du tout une partie inerte du tissu cérébral.

C'est certainement dans le métabolisme protéique qu'il faut chercher la clef de l'énigme des particularités fonctionnelles du tissu cérébral.

A ce point de vue, la fraction des protéines qui porte le nom de phosphoprotéines et qui se rencontre dans divers tissus de l'organisme animal, y compris le tissu nerveux, mérite qu'on lui accorde une attention particulière. Ce qui prouve que les phosphoprotéines exercent des fonctions importantes dans le cerveau, c'est avant tout le renouvellement très rapide de leur phosphore — cela a été établi au cours des essais faits avec le phosphore marqué $^{32}P$ (30, 31, 32). L'intensité du métabolisme des phosphoprotéines dépasse de beaucoup celle de l'acide nucléique et des phospholipides.

Dans les recherches sur les coupes de l'écorce de cerveau de rats, Engelhardt (33) a constaté que l'intensité du métabolisme des phosphoprotéines dépasse celle d'autres composés phosphoprotéiques, en particulier de l'acide nucléique ; il a constaté aussi que le métabolisme des phosphoprotéines dépend des processus d'oxydation et est lié en premier lieu au mécanisme de phosphorylation oxydative.

Les substances protéiques du cerveau ne sont pas seulement liées aux acides nucléiques : elles forment des composés avec diverses autres substances : lipoïdes, cholesterol, hydrates de carbone (glycogène).

Quoique la présence des lipoprotéines dans le tissu nerveux soit tout à fait possible, leur étude n'a guère été entreprise.

Folch (34) a réussi à extraire du cerveau des « protéolipides » solubles dans les solvants organiques (avant tout dans le chloroforme contenant de l'alcool) et qui se distinguent des lipoprotéines par leur grande teneur en lipides. Les « protéolipides » sont des substances extrêmement labiles. Les protéolipides de Folch, extraits du cerveau humain et du cerveau de taureau (35) sont très semblables quant à leur teneur en azote total, phosphore et acides aminés.

*Glucides*

Le tissu nerveux ne dispose pas de grandes réserves de glucides. La quantité de glycogène dans le cerveau est de 70-130 mg. % (36).

Après la découverte de la phosphorylase, qui provoque la dégradation du glycogène dans les muscles (ainsi que dans le foie), on a commencé à nier en général l'existence de l'amylase dans les tissus de l'organisme animal : les anciennes données (37, 38, 39) sur l'amylase dans le cerveau avaient été oubliées. Cependant les essais de Raschba (40) ont démontré la présence d'amylase très active dans le cerveau ; elle est liée aux protéines du cerveau, et s'en sépare au cours de l'autolyse. La dégradation du glycogène dans le cerveau a lieu, avant tout, sous l'influence de l'amylase. La phosphorylase dans le cerveau provoque principalement la synthèse des polysaccharides.

Cori (41) a supposé que le glycogène du cerveau se forme sous l'influence de la phosphorylase (qui forme le polysaccharide non ramifié, du type de l'amylose) et d'un autre ferment complémentaire qui transforme l'amylose en polysaccharide ramifié (glycogène). Dans notre Institut, Hajkina et E. Gontcharova (42) ont réussi à extraire ces deux ferments (phosphorylase et isomérase) du tissu cérébral en les séparant au moyen d'un fractionnement au sulfate ammonique.

Nos recherches sur les ferments qui provoquent la dégradation et la synthèse du glycogène nous ont conduits (43) à mesurer la concentration de glycogène de diverses parties du cerveau et à découvrir sa présence dans l'écorce des grands hémisphères et dans le cervelet d'animaux sains ; nous avons établi, que le glycogène est sujet à des changements perpétuels.

C'est une erreur d'affirmer que le taux de glycogène ne varie pas dans le cerveau, comme le montrent les

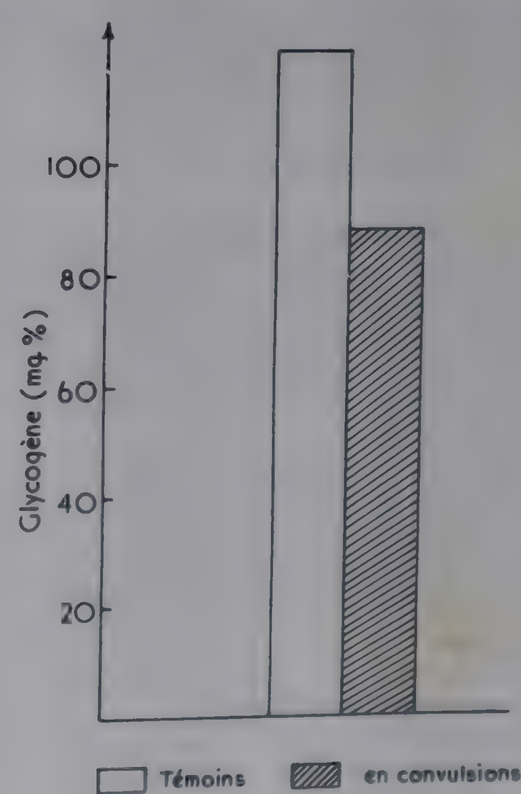


Fig. 6. — Concentration du cerveau de chien en glycogène après excitation suivie de convulsions.

résultats obtenus au cours des recherches sur le métabolisme des hydrates de carbone du cerveau lors de différents états fonctionnels : pendant les convulsions, par exemple, l'activité de l'amylase augmente et la concentration du glycogène diminue (figure 6).

L'intensité de son métabolisme se manifeste dans le fait que la vitesse de renouvellement du carbone marqué du glycogène du cerveau est, d'après Prohorowa (45), égale ou même supérieure à la vitesse de renouvellement du glycogène du foie. La même conclusion peut être tirée des recherches sur les changements dans la teneur en glycogène du cerveau des souris dans différentes conditions (46).

La question de savoir si la glycolyse peut se produire dans le cerveau sans participation du phosphore n'a plus qu'un intérêt historique : il est clair à présent que la glycolyse comme toute réaction génératrice d'énergie ne peut se produire qu'avec la participation du phosphore. On a, de plus, découvert dans les extraits de cerveau les produits intermédiaires qui ont été observés dans les muscles et les mêmes ferments de métabolisme ont été découverts.

Nous avons confirmé les données d'Ochoa (47) et d'autres sur la présence de l'hexokinase dans le cerveau, dans la substance grise et dans la substance blanche (48) ; elle est plus active chez les jeunes animaux (49). Nous avons étudié les propriétés et le rôle d'autres ferments de cerveau, phosphoglucomutase (50), aldolase (51) et adénosinetriphosphatase (52), également étudiés par Gore (53). Nous avons montré que l'hexokinase, l'aldolase, la phosphorylase et l'adénosinetriphosphatase possèdent une plus grande activité dans l'écorce des grands hémisphères et dans le cervelet ; l'intensité de la glycolyse n'est pas la même dans toutes les parties du cerveau.

Le métabolisme oxydatif du glucose commence avec la formation de l'acide pyruvique, qui est oxydé par la voie du cycle des acides tricarboxyliques.

### *Lipides*

Les lipides jouent un rôle considérable dans la structure chimique du tissu nerveux. Presque la moitié de la substance sèche du cerveau en est constituée ; ils sont plus nombreux encore dans la moelle épinière. Les lipides du cerveau se composent par excellence de cholestérine, de glycérine-phosphatides, de sphyngomiéline et de cérébrosides.

La structure des glycérophosphatides n'est pas assez bien étudiée. Le tissu nerveux contient outre les glycérophosphatides, lécithine et céphaline connus depuis bien longtemps, la sérine-céphaline, trouvée par Folch (54), et les acétalphosphatides de Feulgen (55, 56).

Le lipide que Folch (57) a isolé du cerveau et qu'il a appelé strandine est très riche en glucides. Klenk (58) suppose qu'il est un ganglioside.

Afin d'étudier plus profondément les stérides du cerveau Poliakova (59) dans notre Institut a étudié la composition de la fraction non saponifiée du cerveau d'animaux, de la substance blanche et de la substance grise des grands hémisphères du cerveau de l'homme ; la séparation de ces composés a été effectuée d'après la méthode d'adsorption chromatographique. Les recherches sur le cerveau de l'homme ont montré que la fraction non saponifiée de la substance blanche des grands hémisphères contient 93 % de stérides (85 % pour la substance grise) ; il est à noter que dans ces deux cas la masse essentielle des stérides est représentée par le cholestérol ; la substance grise contient du 7-oxycholestérol.

Les expériences *in vitro* ont démontré que les coupes de cerveau peuvent synthétiser des phospholipides (60). La synthèse du cholestérol a lieu seulement dans les coupes de cerveau des rats nouveau-nés ; les coupes de cerveau des rats adultes ne peuvent pas synthétiser le cholestérol (61). La synthèse de cholestérine peut aussi être observée au cours d'expériences *in vitro* ; la synthèse diminue avec la croissance des rats (62).

Ces derniers temps ont paru plusieurs recherches consacrées à l'isolement de divers lipides des différentes parties du cerveau et à l'étude, à l'aide de radiophosphore, de la vitesse de pénétration du phosphore dans divers lipides (63).

Le système nerveux central ne constitue pas un tissu homogène. Il contient outre beaucoup de types de neurones ayant des processus métaboliques différents, un grand nombre de cellules gliales et d'autres éléments. Ce fait signifie que les systèmes fermentaires et les processus métaboliques dans les cellules individuelles et dans les sections tissulaires isolées doivent être soumis à une étude plus détaillée. Sous ce rapport les recherches biochimiques et morphologiques de Flexner (64), Bodian (65) et Pope (66) présentent une importance considérable. A ce genre de recherches se rapportent les essais récemment entrepris par Aboad (67), qui ont réparti en quatre fractions la substance grise du cerveau et la substance blanche de la moelle épinière : fraction de noyaux, de mitochondries, solution surnageante et lipides. Ils ont étudié le taux des diverses substances dans ces fractions (acides nucléiques et plusieurs substances contenant du phosphore) et l'activité des ferments (ferments d'oxydation, de phosphorolyse et de glycolyse).

### *Biochimie comparée du cerveau*

Les recherches de biochimie comparée, c'est-à-dire l'étude des processus chimiques dans le cerveau au cours de leur développement ontogénétique et phylogénétique est une des voies qui permet d'étudier la biochimie fonctionnelle du système nerveux central.

L'étude biochimique du cerveau en ontogénèse préoccupe Kreps dès 1946 (68, 69) et ses collaborateurs à l'Institut de Physiologie I. Pavlov (Leningrad). Kreps a étudié le développement de l'activité de certains systèmes fermentaires du cerveau et a comparé le développement biochimique à la maturation fonctionnelle du système nerveux central. Il a établi que chez les Mammifères les plus évolués l'activité fermentaire atteint son maximum dans l'écorce des grands hémisphères (70, 71, 72). L'activité de l'anhydrase carbonique est la plus élevée dans les parties du cerveau qui sont au point de vue fonctionnel les plus importantes.

Chez les Vertébrés plus développés l'intensité de la respiration s'accroît et l'intensité de la glycolyse anaérobie baisse (73). Parallèlement, on constate qu'au cours de l'évolution des Vertébrés l'activité de la cytochrome-oxydase et de tout le système cytochrome du tissu cérébral va en croissant (74).

Chez les Mammifères et les Oiseaux à l'état adulte l'activité des systèmes fermentaires atteint son maximum dans l'écorce des grands hémisphères, où elle dépasse de beaucoup leur activité dans la moelle épinière. Le cervelet se caractérise par une activité élevée qui souvent n'est pas inférieure à celle de l'écorce (75, 76). Ces résultats confirment nos données sur l'activité maximale des ferments du métabolisme glucidique dans l'écorce des grands hémisphères (49, 50, 51, 52).

Les variations de l'activité de la cholinestérase dans le cerveau et la moelle épinière de jeunes rats au cours de leur développement ont été étudiées par Bayliss (77).

Bieth (78, 79) a entrepris une recherche comparative de la composition du cerveau des animaux adultes de quelques espèces de Vertébrés ; il a aussi étudié les composés phosphorés acidosolubles du cerveau de rats à des périodes différentes de leur développement (de 3 jours jusqu'à un an).

Skvirskaja et Silitch (22) ont montré que le taux des acides nucléiques dans le cerveau est très élevé au cours des premiers stades du développement embryonnaire et qu'il baisse graduellement avec l'âge de l'embryon ; après la naissance la cadence de cet abaissement s'affaiblit et à l'âge d'un mois il atteint une valeur proche de celle du cerveau des animaux adultes.

Le taux des phosphoprotéines est également plus élevé au cours des premiers stades du développement ; il baisse légèrement chez les embryons âgés de 9 jours et à l'âge d'un an, atteint la valeur que l'on trouve chez les animaux adultes (voir tableau II).

Les modifications de l'activité de la désoxyribonucléase coïncident avec l'apparition de nouvelles fonctions : l'activité de la désoxyribonucléase monte vers le 20e jour du développement embryonnaire, ce qui est probablement lié à la différenciation renforcée des organes. Une deuxième montée de l'activité s'observe vers le 9e jour du développement postnatal, c'est-à-dire au début de la fonction visuelle.

TABLEAU II

*Le taux des acides nucléiques et des phosphoprotéines dans le cerveau de lapins à différents âges*

| Age | Phosphore | | |
|---|---|---|---|
| | RNA | DRNA | PP |
| Embryons : | | | |
| 16-20 jours | 416 | 368 | 20.8 |
| 26-29 jours | 321 | 140 | 31.0 |
| Nouveau-nés | 298 | 148 | 21.7 |
| Lapins jeunes : | | | |
| 9-10 jours | 236 | 89 | 18.0 |
| 1 mois | 195 | 48 | 3.7 |
| Lapins adultes | 166 | 37 | 6.7 |

Les résultats sont exprimés en mg. % du poids sec.

### *Métabolisme et fonction*

Le cerveau se caractérise par un métabolisme très actif. A l'état de veille la température du cerveau est 0.5° C. plus élevée que la température du sang artériel. Le cerveau d'un homme adulte absorbe environ un quart de la quantité totale d'oxygène que consomme un homme (80).

Les processus métaboliques du cerveau ne s'interrompent jamais, vu que son activité se poursuit toujours. L'activité métabolique du cerveau qui change continuellement dépend des variations de son activité fonctionnelle. Les plus grands changements dans l'activité du cerveau et dans son métabolisme s'observent dans les conditions expérimentales réalisées au cours des états d'excitation et d'inhibition. C'est pourquoi l'influence de ces deux états sur le métabolisme du cerveau a si souvent été étudiée.

Les recherches ont montré par exemple qu'en cas de fonction affaiblie (sommeil ou narcose) les composés phosphorés riches en énergie commencent à s'amasser, en cas d'activité intense ils se dégradent (augmentation de glycolyse et formation intense d'acide lactique). Olsen et Klein (81) ont observé qu'en cas de convulsions provoquées par le courant électrique, le taux de glucose, de glycogène, d'acide adénosinetriphosphorique et de phosphocréatine diminue dans le cerveau de chat, le taux de phosphore inorganique et d'acide lactique s'accroît. Le taux d'acide adénosinetriphosphorique change moins chez les rats que chez les chats. Le taux d'acide lactique augmente aussi en cas de convulsions provoquées par injection de camphre et de strychnine (82).

La dégradation de la phosphocréatine au cours de l'excitation est très rapide, tandis que la quantité d'ATP diminue à peine, étant continuellement reconstituée aux dépens de la phosphocréatine. Dans la période de 15-30 secondes qui suit l'excitation électrique la teneur en phosphocréatine revient progressivement à la normale (83).

Les expériences à l'aide de narcotiques ont mis en évidence le niveau élevé de phosphocréatine et d'ATP et le taux infime de l'acide lactique dans le cerveau : on voit donc que les changements qui se produisent au cours de la narcose sont l'inverse de ceux qui suivent les convulsions. Les narcotiques inhibent la désaminase de l'acide adénylique (84).

Dawson et Richter ont observé au cours d'expériences avec $^{32}P$ un ralentissement de la synthèse des nucléoprotéines et des phospholipides dans le cerveau des rats anesthésiés au nembutal (85). Le taux de l'acide lactique dans le cerveau des rats diminue aussi pendant le sommeil (86).

McIlwain et coll. ont étudié, *in vitro*, le métabolisme du cerveau excité en appliquant la méthode d'excitation des coupes de cerveau par le courant électrique (87) ; Ayres et McIlwain ont décrit des appareils spéciaux à cette fin (88). Les recherches ont montré que l'excitation des coupes de cerveau (de même que l'excitation *in vitro*)

augmente la respiration et la formation d'acide lactique (89), diminue le taux de phosphocréatine et augmente le taux de phosphate inorganique (90) : son influence est semblable à celle du 2,4-dinitrophenol.

McIlwain a fait des expériences à l'aide de coupes produisant les conditions d'une hypoglycémie prononcée et a étudié son influence sur le métabolisme du tissu cérébral. Le tissu cérébral s'épuise et ne réagit pas à l'excitation électrique ; sa respiration ne s'accélère pas lorsqu'on ajoute du glucose dans le milieu ; il conserve pourtant la faculté d'accroître sa production d'acide lactique après excitation (91). En étudiant la respiration et la glycolyse dans les coupes du cerveau d'un homme et d'un cobaye, en présence de concentrations différentes de glucose dans le milieu et sous l'influence de stimuli électriques, McIlwain (92) a constaté une augmentation de la respiration.

Heald (93) a étudié la transformation de la phosphocréatine dans les coupes d'écorce stimulées électriquement et a constaté que la phosphocréatine se dégrade à la vitesse de 1400 m-moles/g./heure après une période latente de 2-3 secondes ; la dégradation dure 5 minutes et a été précédée d'une faible baisse du niveau de l'adénosinetriphosphate. Si les stimuli électriques cessent après 7 secondes, la phosphocréatine se resynthétise et rejoint son niveau initial en 20 secondes.

Plusieurs savants se sont intéressés à la question de la formation de l'ammoniaque dans le cerveau lors de l'excitation et de la dépression de l'activité nerveuse. Vladimirova (94, 95) a établi en 1938 que l'excitation du système nerveux central due au camphre augmente la formation de l'ammoniaque (et de l'acide lactique), et inversement la dépression, provoquée par l'uréthane, est accompagnée d'une diminution du taux de l'ammoniaque dans le cerveau. Toute excitation inconditionnelle (courant électrique), ainsi que l'excitation réflexe, produit également une augmentation du taux de l'ammoniaque dans le cerveau (96). Inversement, toute action qui provoque un état d'inhibition fait diminuer le taux d'ammoniaque dans le cerveau.

Richter et Dawson (1948) ont établi aussi (97) que le taux d'ammoniaque dans le cerveau augmente lors des convulsions, qu'elles soient provoquées par l'injection de picrotoxine, par une excitation électrique ou par l'anoxie ; il diminue au cours de l'anesthésie par le nembutal.

Le système adénylique n'est pas l'unique source de formation de l'ammoniaque (98), comme on le constate dans le tissu musculaire. La formation de l'ammoniaque dans le cerveau a lieu aussi à partir de la glutamine qui perd alors son groupe amidé et se transforme en acide glutamique. Au cours de la période de renouvellement qui suit l'excitation, l'ammoniaque est éliminé et la glutamine augmente (99, 100). Ainsi, le système glutamine-acide glutamique participe à la régulation du taux de l'ammoniaque dans le tissu cérébral.

### *Métabolisme du cerveau après excitation de l'activité nerveuse*

Nos études ont porté pendant plusieurs années (101, 102) sur l'étude du métabolisme cérébral à différents états fonctionnels et nous venons d'en exposer certains résultats ; mes collaborateurs et moi avons eu notre attention particulièrement attirée sur l'étude des processus métaboliques du cerveau après excitation ou inhibition de l'activité nerveuse (103, 104, 105, 106).

Pavlov a maintes fois indiqué que les processus fondamentaux qui caractérisent l'activité nerveuse supérieure sont les processus d'excitation et d'inhibition et que leur interprétation dépend avant tout de l'étude de la physique et de la chimie du système nerveux (107).

Au cours de nos recherches sur le métabolisme après excitation ou inhibition de l'activité nerveuse, nous avons considéré que ces états peuvent être provoqués par différents moyens utilisés pour engendrer tel ou tel état du système nerveux, qui peuvent ainsi être à l'origine de certaines modifications des processus métaboliques. Nous avons cependant estimé qu'il devait être possible d'établir les particularités principales du métabolisme cérébral après l'excitation ou inhibition en nous servant tout d'abord des agents pharmacologiques pour passer ensuite, ayant établi certaines caractéristiques d'ordre général, à l'emploi des stimulants physiologiques et des stimulants non-conditionnels et conditionnels.

Nous avions déjà eu recours aux stimulants physiologiques au commencement de nos études dans le domaine de la biochimie du cerveau, quand Gorodisskaya (108) étudia l'influence des stimulations naturelles sur les processus de la protéolyse dans la zone visuelle de l'écorce des grands hémisphères et établit que le passage des centres visuels à l'état d'activité plus élevée est liée à l'intensification des processus du métabolisme protéique.

Nos recherches ont porté sur l'étude du métabolisme des acides nucléiques, des glucides, de l'acide adénosinetriphosphorique, des phosphoprotéines et des phospholipides. Les expériences ont été effectuées sur le lapin, le chien et le rat. Pour éviter la dégradation des composés phosphorés labiles à la suite de l'irritation produite par la décapitation, nous avons injecté avant la décapitation 1 ml. d'une solution d'hexenal à 10 %, et le cerveau a été congelé dans l'air liquide. Nous avons provoqué l'excitation par injection de pervitine (qui, à l'instar de

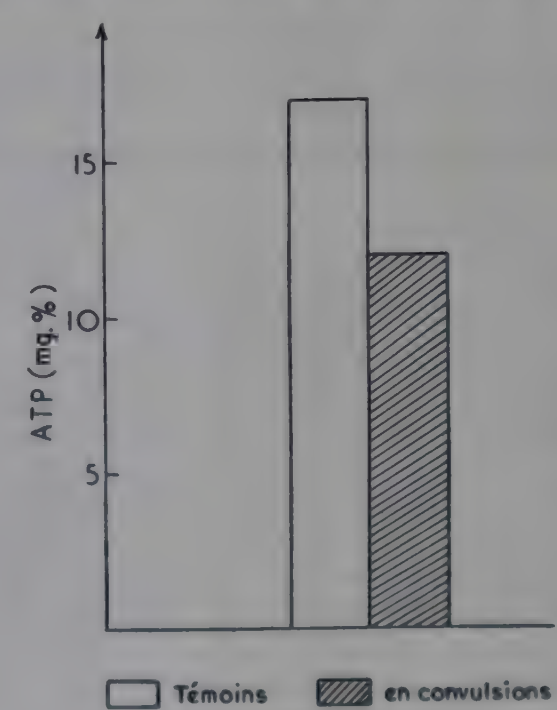


FIG. 7. — Concentration du cerveau de rat en ATP après excitation suivie de convulsions.

la benzédrine, est largement employée comme stimulant du système nerveux) et de cardiazol qu'on utilise aussi assez couramment.

Nous avons étudié tout d'abord l'influence de l'excitation prolongée (provoquée par l'injection de fortes doses de cardiazol ou par la stimulation électrique) causant des convulsions. Dans ce cas, le taux du glycogène et de l'acide adénosinetriphosphorique dans le cerveau baisse (109, 110) ; nos données (figures 6 et 7) confirment donc les résultats des recherches d'Olsen et Klein (81) et d'autres chercheurs (111, 1).

Au cours d'expériences de nature plus physiologique, l'excitation a été produite par une seule injection de pervitine (5 à 7 mg./kg.) ou de cardiazol (50 à 70 mg./kg.) 4 heures avant que les animaux aient été sacrifiés ; nous avons constaté que les modifications du métabolisme cérébral sont différentes suivant la substance injectée (109).

Dans l'excitation provoquée par la pervitine, le taux de l'acide lactique préformé diminue par rapport à la normale (figure 8), tandis qu'il est plus élevé après injection de cardiazol (112). Le taux d'ATP et de glycogène (figure 8) après excitation causée par la pervitine est

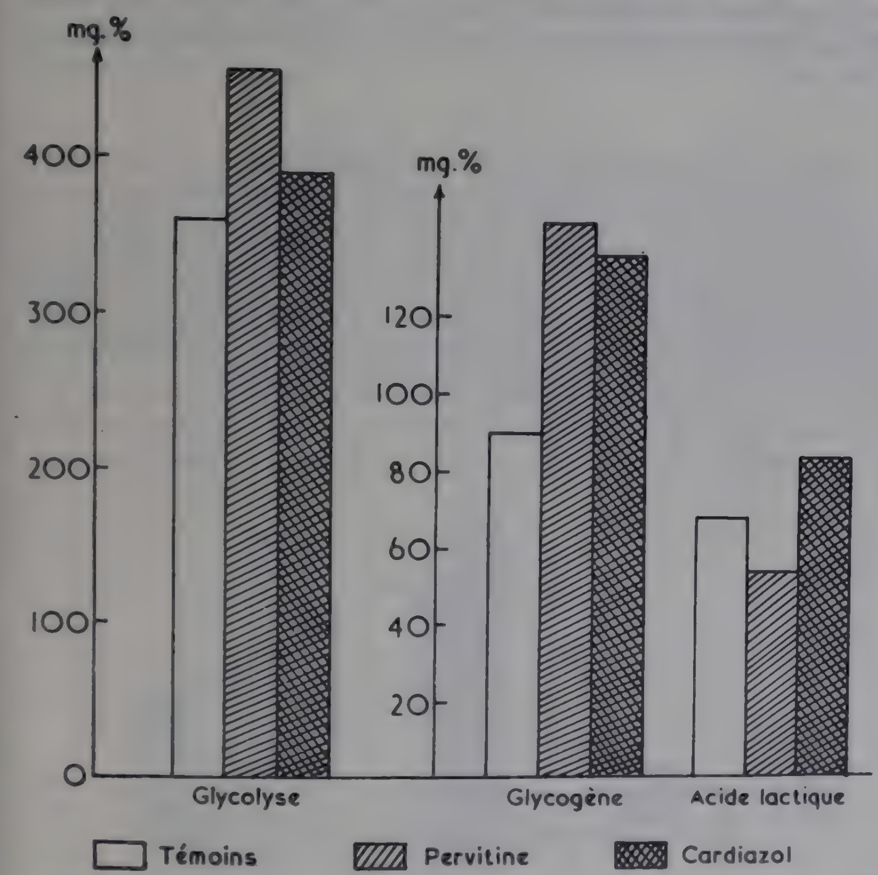


**Fig. 8.** — **Influence de l'excitation (pervitine ou cardiazol) sur la teneur du cerveau de lapin en glycogène et en acide lactique et sur l'intensité de la glycolyse.**

augmenté, tandis que dans l'excitation provoquée par le cardiazol le taux d'ATP est très voisin de la normale.

Dans l'excitation provoquée par la pervitine le taux d'acide ribonucléique ne change presque pas tandis qu'apparaît une faible augmentation du taux de l'acide désoxyribonucléique (113).

L'étude des phospholipides au cours de l'excitation due à la pervitine a montré (114), que pendant les trois premières heures qui suivent l'administration de pervitine il n'y a pas de changements notables du taux total des phospholipides, ni du taux des phospholipides saturés et non-saturés.

Toutefois, grâce à l'emploi de phosphore radioactif il a été possible d'établir que l'incorporation du phosphore dans les deux fractions des phospholipides, lors de l'excitation due à la pervitine, s'effectuait autrement qu'à l'état normal : les modifications de l'état fonctionnel du système nerveux entraînent des modifications du métabolisme des phospholipides. Nos recherches ont montré que les modifications du métabolisme cérébral sont différentes suivant la substance employée pour provoquer l'excitation, ce qui explique les différences d'effet physiologique exercé par ces substances : la pervitine intensifie les processus du métabolisme des glucides et contribue à l'accumulation d'une substance à haute activité biochimique et physiologique, l'ATP, qui est responsable de l'augmentation de la capacité de travail du système nerveux sous l'influence de pervitine. Le cardiazol, par contre, provoque l'excitation de l'écorce cérébrale sans augmenter sa capacité de travail (figure 9).

Vladimiroff (115) a étudié l'influence de l'excitation du système nerveux central sur la vitesse du renouvellement de l'acide ribonucléique et des phospholipides. L'excitation a été provoquée par la stimulation pendant trois heures (avec un intervalle de repos) des récepteurs de la peau chez les rats par le courant électrique. Les recherches ont montré que l'état d'excitation du cerveau entraîne, à en juger par les résultats de la détermination de l'activité spécifique relative du phosphore, une augmentation de l'intensité du renouvellement du phosphore de l'acide ribonucléique et des phospholipides ; l'intensité du métabolisme de l'acide ribonucléique augmente de 20 % ; celle du métabolisme des phospholipides devient une fois et demie plus grande.

Considérant que le caractère de l'excitation peut différer selon la nature de la substance excitante et la durée de son action sur le système nerveux, nous avons étudié le métabolisme de l'ATP dans le cerveau à des moments différents après injection de pervitine ou du cardiazol (1, 2 et 4 heures).

Nos recherches ont montré (116), que lors de l'excitation provoquée par la pervitine, le taux de l'ATP dans le cerveau 1 heure après injection de pervitine baisse pour s'élever ensuite, de sorte que 2 heures après l'injection de pervitine le taux de l'ATP revient à la normale et après 4 heures dépasse considérablement le niveau normal (figure 9). Ainsi, ces données confirment les résultats des recherches exposées aux paragraphes précédents au cours desquelles le taux de l'ATP dans le cerveau avait été mesuré 4 heures après l'injection de pervitine. Ces données permettent en outre de conclure que le métabolisme de l'ATP est différent selon la période de l'excitation. Les variations du taux de phosphore inorganique se font en sens inverse.

Après excitation provoquée par le cardiazol la situation est différente : 1 heure après l'injection du cardiazol, le taux de l'ATP augmente, puis est suivi d'une chute, de sorte que 2 heures et surtout 4 heures après l'injection du cardiazol le taux d'ATP est inférieur à la normale. Les variations du taux de phosphore inorganique se font aussi en sens inverse (figure 9).

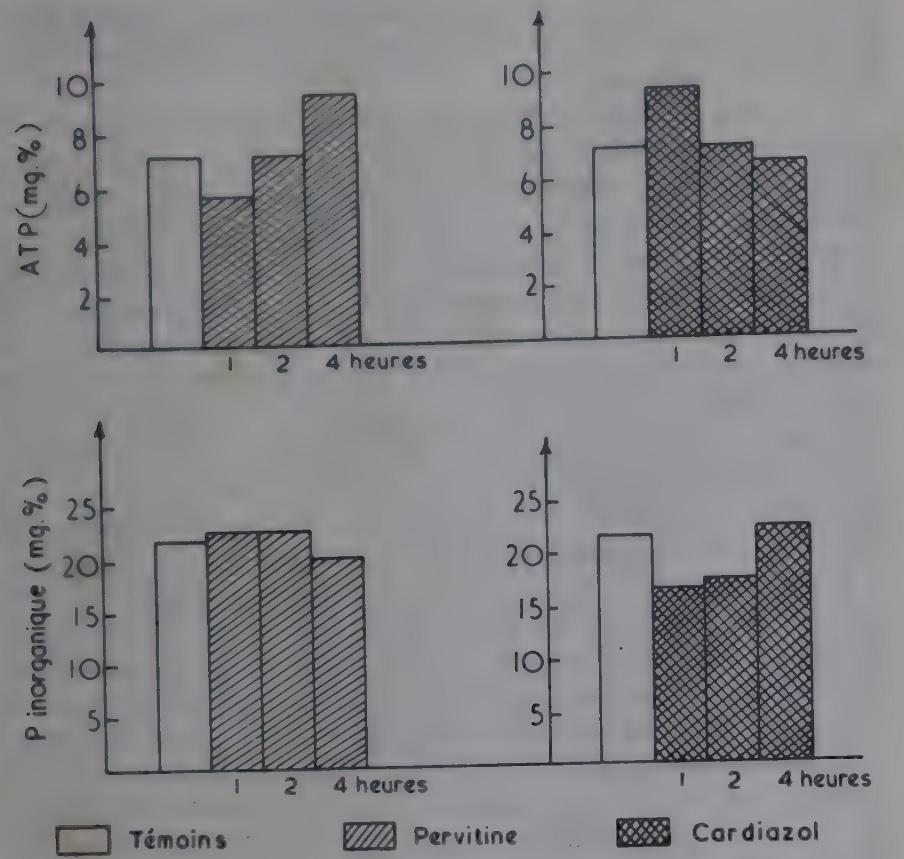


FIG. 9. — Influence de l'excitation (pervitine ou cardiazol) sur la teneur en ATP et en phosphore inorganique au cours des 4 premières heures suivant l'injection.

Ces recherches ont montré une fois de plus que la pervitine et le cardiazol qui possèdent des effets physiologiques différents, ont une influence différente sur le métabolisme de l'ATP dans le cerveau.

Nous avons ensuite effectué des recherches sur des animaux soumis à une excitation chronique provoquée par l'action prolongée du courant électrique, ou par l'interruption du sommeil physiologique. Dans le premier cas, les rats placés dans une cage spéciale à électrodes ont été soumis à l'action prolongée (pendant 1-1½ mois) quotidienne d'un courant électrique de 25 à 40 volts. Dans le dernier cas, les rats ont été placés pendant 3 jours dans un tambour qui tournait 30 secondes avec des arrêts de 5 minutes, de sorte, que les animaux ont été privés du sommeil durant 3 jours.

Les expériences ont montré (117), que l'insomnie chronique provoque une certaine diminution de l'intensité de la glycolyse ; le taux du glycogène ne change presque pas ; le taux de l'ATP baisse (figure 10). L'excitation par le courant électrique durant plusieurs jours provoque également une diminution du taux de l'ATP.

L'étude du métabolisme des acides nucléiques au cours d'excitation prolongée provoquée par le courant électrique a montré (22) que le taux des acides nucléiques et des phosphoprotéines ne change presque pas.

L'utilisation du phosphore marqué nous a permis d'établir que malgré l'absence de changements du taux des acides nucléiques leur métabolisme dans l'excitation chronique change, notamment la vitesse du renouvellement de l'acide nucléique baisse (22).

Poursuivant les recherches du métabolisme cérébral pendant l'exaltation des processus nerveux, Haikina et Kratchko (118) ont étudié le métabolisme lors de la « rupture » de l'activité nerveuse, provoquée par la « collision » entre deux stimulations opposées : une stimulation réflexe conditionnelle alimentaire (sonnerie) et une stimulation non-conditionnelle (courant électrique). L'élaboration des réflexes conditionnels et la « rupture » de l'activité nerveuse ont été effectuées d'après la méthode de Gorcheleva (119).

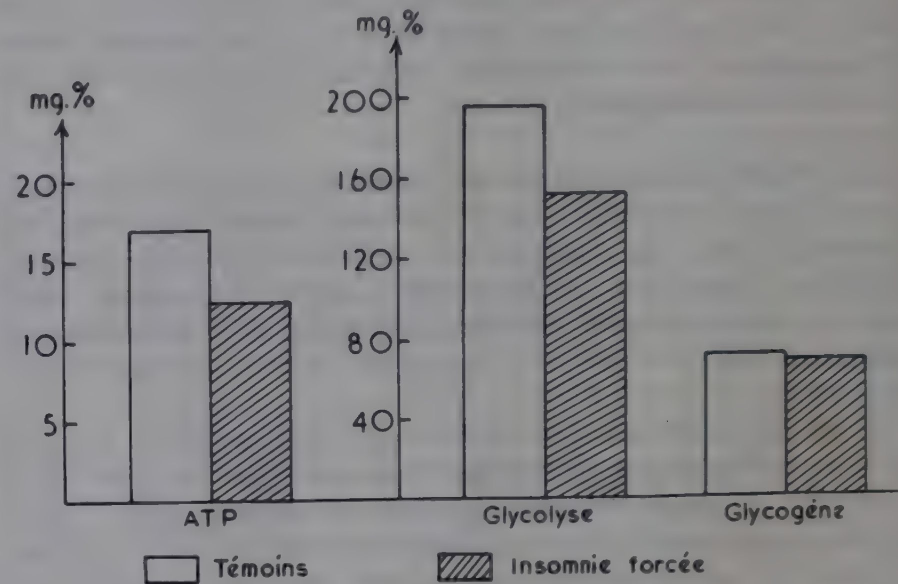


FIG. 10. — Influence de l'insomnie forcée sur la teneur en ATP et en glycogène du cerveau de rat et sur l'intensité de sa glycolyse.

Après l'élaboration du réflexe conditionnel alimentaire moteur à la sonnerie, et au moment même de la sonnerie, les rats ont été soumis à l'action d'un courant électrique de 20 à 30 volts pendant 10 secondes. 8 à 10 jours après action combinée de ces deux stimulants le réflexe conditionnel disparaît : les rats entendant la sonnerie se cachent immobiles dans un coin de la cage.

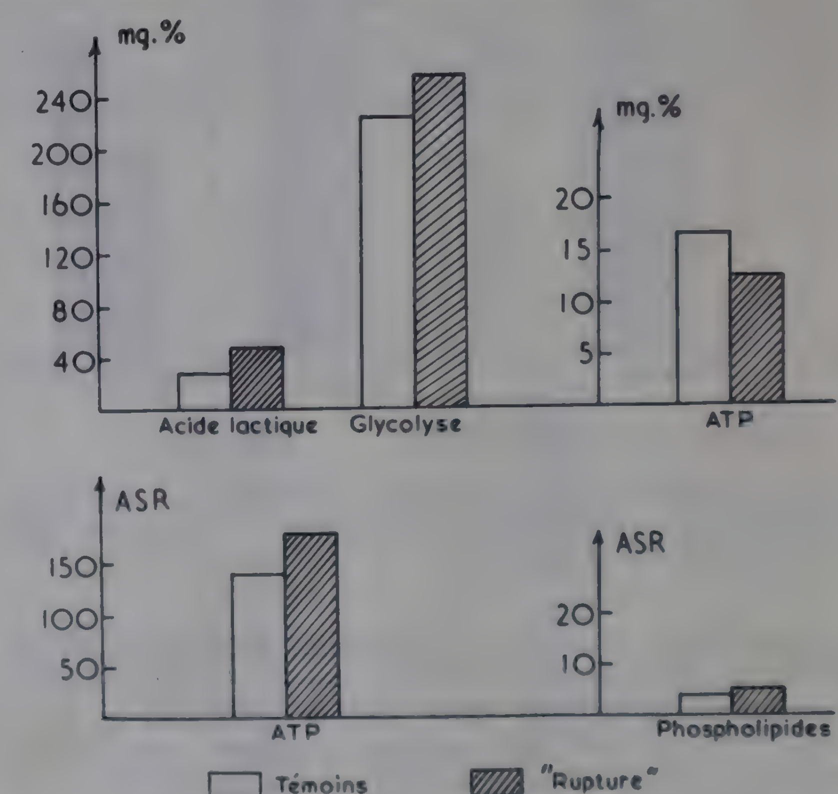


FIG. 11. — Influence de la « rupture » de l'activité nerveuse sur le taux d'acide lactique et d'ATP, sur la glycolyse et sur la vitesse du renouvellement de l'ATP et des phospholipides dans le cerveau du rat. ASR = activité spécifique relative.

Les recherches entreprises sur le métabolisme cérébral ont montré que chez ces rats le taux de l'ATP diminue tandis que le taux de phosphore inorganique augmente ; on constate en outre une augmentation du taux de l'acide lactique et une augmentation de l'intensité de la glycolyse (figure 11). La détermination de l'activité spécifique relative après l'injection de $^{32}P$ a montré que la vitesse du renouvellement du phosphore de l'ATP augmente.

Il y a tout lieu de croire que dans les conditions d'expérience exposées ci-dessus (dans la « rupture ») se produit une surtension extrême, épuisante de l'activité nerveuse.

L'état d'excitation du système nerveux provoqué par les stimulants proches des stimulants physiologiques produit donc une certaine augmentation, parfois très faible, du taux de l'ATP et du glycogène, une élévation du taux du phosphore inorganique et une glycolyse plus intense. Il entraîne en outre à en juger par l'accroissement des activités spécifiques relatives, une augmentation de l'intensité du métabolisme de l'acide ribonucléique, des phosphoprotéines et des phospholipides.

Si les stimulants sont trop forts et si leur action est assez prolongée, la nature des changements du métabolisme cérébral est susceptible de subir certaines modifications : l'épuisement du système nerveux peut survenir ou même un passage de l'état d'excitation à l'état d'inhibition peut avoir lieu.

### *Métabolisme du cerveau après inhibition de l'activité nerveuse*

Dans nos recherches sur le métabolisme du cerveau après inhibition de l'activité nerveuse, nous nous sommes servis des agents pharmacologiques qui provoquent le sommeil narcotique, plus précisément du médinal sodique ou de l'amytal sodique à des doses provoquant un sommeil narcotique voisin du sommeil naturel, les animaux réagissant parfaitement au bruit, au contact, etc.

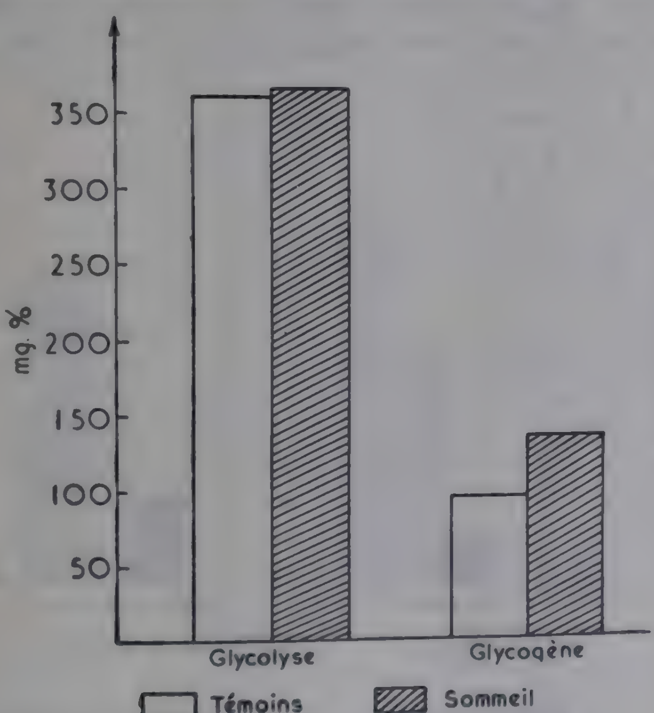


FIG. 12. — Influence du sommeil narcotique (4 heures) sur la teneur en glycogène et sur l'intensité de la glycolyse du cerveau de lapin.

Les recherches sur le métabolisme cérébral des glucides chez le lapin en sommeil narcotique pendant 4 heures ont montré que l'intensité de la glycolyse était élevée et ne différait que très peu de la normale ; le taux du glycogène était augmenté, ce qui suggère que le métabolisme des glucides est assez actif, mais que leur utilisation est probablement diminuée (figure 12). Le taux de l'ATP pendant le sommeil narcotique (109) monte (figure 13).

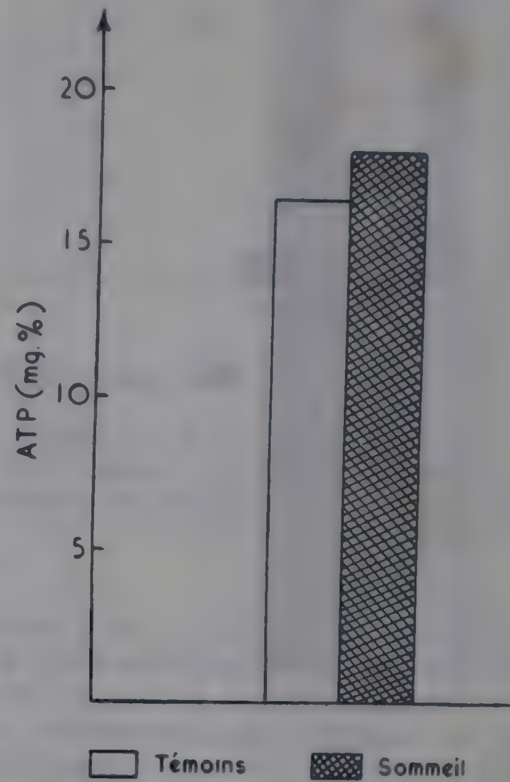


FIG. 13. — Influence du sommeil narcotique (4 heures) sur la teneur en ATP du cerveau de rat.

Quant au métabolisme cérébral des acides nucléiques, on a constaté que leur taux chez le rat ne subissait pas de variations considérables pendant le sommeil. En même temps, l'activité de la désoxyribonucléase augmente d'autant plus que le sommeil est prolongé.

Nos recherches (120) ont aussi porté sur l'intensité d'incorporation de $^{32}P$ dans l'acide ribonucléique, dans les phosphoprotéines et dans les phospholipides du cerveau de rats soumis au sommeil narcotique provoqué par l'uréthane et le médinal (durant 24 heures) et ont montré, que l'activité spécifique relative du phosphore de l'acide ribonucléique baissait de 27.6 %, des phosphoprotéines de 19.2 % et des phospholipides de 22.8 % : il est évident que dans le sommeil narcotique la vitesse du renouvellement de l'acide ribonucléique, des phosphoprotéines et des phospholipides est diminuée (figure 14).

Des résultats analogues ont été obtenus par Vladimirov (115) qui, en se servant du radiophosphore, a étudié l'influence du sommeil narcotique (par hexanastabe ou amytal) sur l'intensité du renouvellement du phosphore des phospholipides et de l'acide ribonucléique dans le cerveau des rats. Il a pu constater une diminution de la vitesse du renouvellement de l'acide ribonucléique et des phospholipides. Il faut noter que dans la majorité des cas le sommeil provoqué par l'amytal était plus profond et les modifications de la vitesse du renouvellement étaient plus accusées.

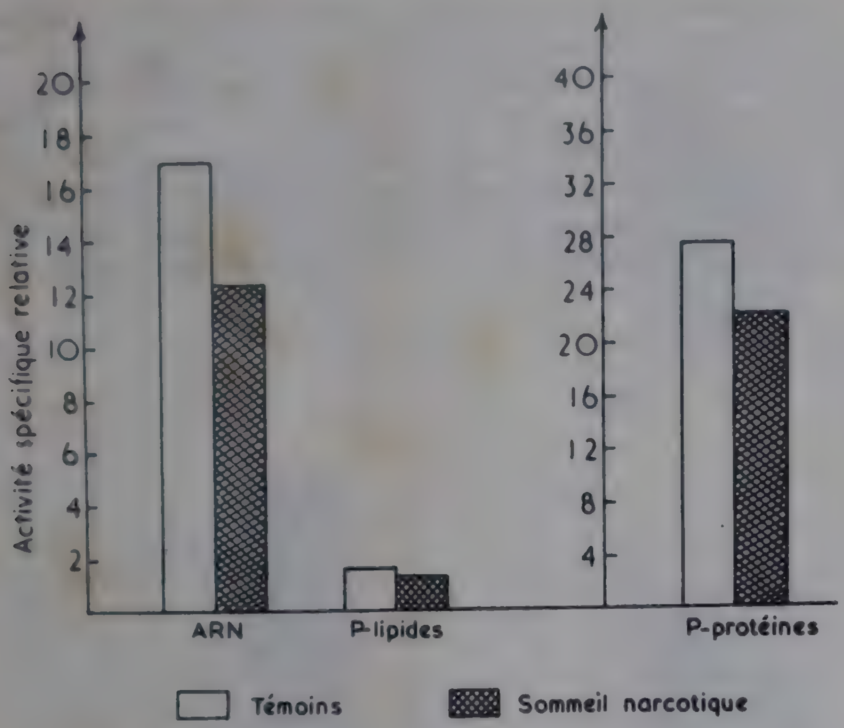


FIG. 14. — Influence du sommeil narcotique (24 heures) sur l'activité spécifique relative de l'acide ribonucléique, des phospholipides et des phosphoprotéines du cerveau de rat tué deux heures après injection de $^{32}P$.

Le sommeil narcotique n'exerce pas d'influence appréciable sur le taux des phospholipides dans le cerveau.

Or, dans l'état d'inhibition provoqué par les agents narcotiques, on constate une diminution des activités métaboliques du cerveau : le taux d'ammoniaque baisse, l'intensité des processus d'oxydation de même que l'intensité de la glycolyse et du renouvellement du glycogène diminue, la vitesse du renouvellement du phosphore des phospholipides et de l'acide ribonucléique est diminuée. Le taux de l'ATP augmente, ce qui peut être considéré comme la préparation pour le travail après le sommeil.

Selon Pavlov l'inhibition est un processus qui protège les cellules nerveuses contre l'épuisement et contribue à leur restauration. Nos recherches permettent de conclure que quoique le métabolisme soit ralenti pendant le sommeil narcotique, son intensité reste assez considérable, créant des conditions où prédominent les processus de synthèse qui assurent la restauration de la capaeité de travail du cerveau.

Ces modifications ayant été observées pendant le sommeil narcotique de courte durée, il a paru intéressant d'étudier l'effet du sommeil narcotique prolongé, par exemple, durant 96 heures. Pour élucider cette question, Haikina et Kratchko (118) ont étudié le métabolisme cérébral chez les rats pendant le sommeil narcotique de différente durée (4, 24, 48 et 96 heures) provoqué par l'injection sous-cutanée d'un mélange d'uréthane et de médinal (2 fois par jour). Leurs expériences ont montré, que le sommeil prolongé provoque dans le cerveau des modifications qui diffèrent des modifications provoquées par le sommeil de courte durée : le taux d'acide lactique après un sommeil de 96 heures au lieu d'être diminué, est augmenté (figure 15) ; le taux d'ATP n'est pas augmenté. On peut penser que ces altérations du métabolisme cérébral pendant le sommeil narcotique prolongé sont dues à l'effet toxique lié à l'influence prolongée des agents pharmacologiques : l'aspect maladif des animaux confirme cette supposition.

Admettant que lors de l'hibernation, il y a une inhibition prolongée du système nerveux central, Skvirskaja et Silitch (120) ont étudié l'inhibition prolongée en utilisant des hibernants (spermophiles). Les recherches ont été effectuées chez les spermophiles en dehors de la période d'hibernation, chez les spermophiles en léthargie hibernale et pendant le réveil forcé (4 heures avant l'expérience), de même que chez les spermophiles à l'état du sommeil pharmacologique durant 24 heures.

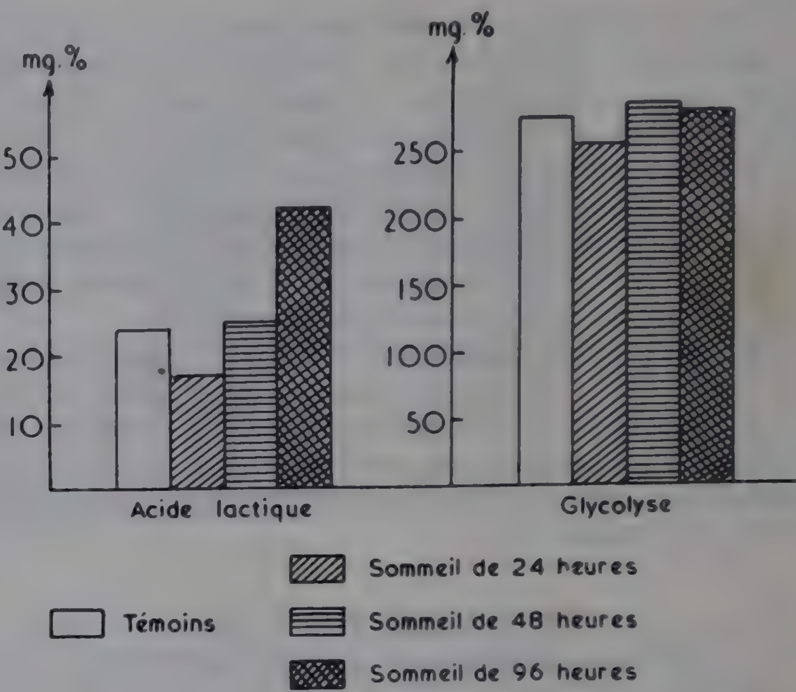


FIG. 15. — Influence du sommeil narcotique de plus longue durée sur le taux d'acide lactique préformé et l'intensité de la glycolyse du cerveau de rat.

Les recherches ont montré des différences considérables, quant à l'incorporation du $^{32}P$ dans l'acide ribonucléique, les phosphoprotéines et les phospholipides du cerveau et de la moelle épinière, entre les animaux réveillés et les animaux en léthargie hibernale, tandis

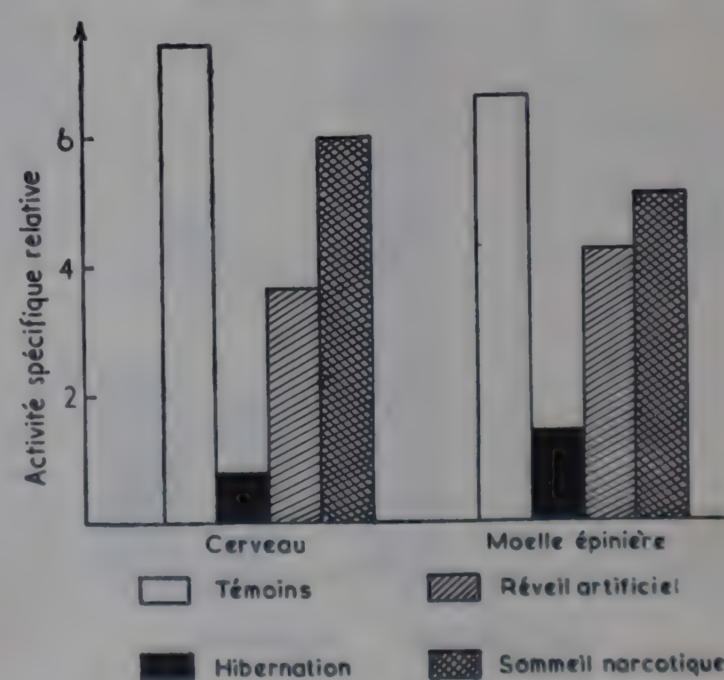


FIG. 16. — Activité spécifique de l'acide ribonucléique, des phospholipides et des phosphoprotéines de cerveau de spermophiles tués 4 heures après injection de $^{32}P$.

que les modifications du taux de ces composés ont été beaucoup moins accusées. L'activité spécifique (le nombre de coups/mg. de phosphore d'une fraction donnée) de divers composés phosphorés dans le cerveau et dans la moelle épinière des spermophiles lors du sommeil hibernal était plusieurs dizaines de fois inférieure à celle d'animaux réveillés ; l'activité spécifique de quelques-uns des composés phosphorés était pratiquement nulle (figures 16 et 17).

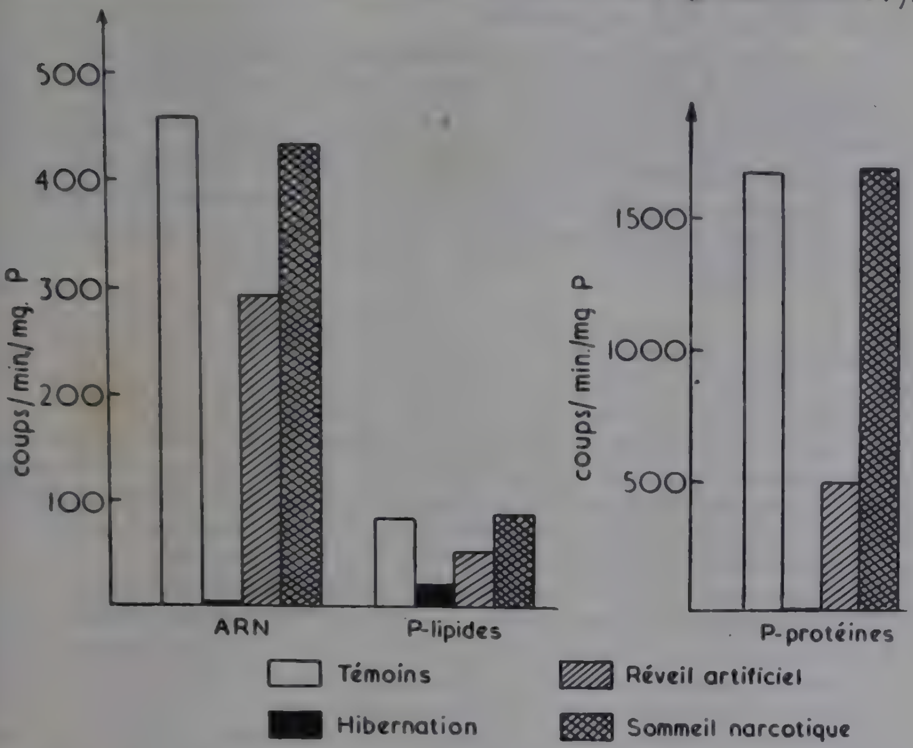


FIG. 17. — Activité spécifique de l'acide ribonucléique, des phospholipides et des phosphoprotéines de la moelle épinière de spermophiles tués 4 heures après injection de $^{32}P$.

Pendant le sommeil narcotique (médinal sodique) de 24 heures, l'intensité de l'incorporation du $^{32}P$ dans les composés phosphorés du cerveau s'effectuait dans le même sens que lors du sommeil hibernal, mais le degré d'abaissement de l'intensité était beaucoup moins accusé.

Les données obtenues chez les animaux à l'état de réveil forcé sont particulièrement intéressantes. Lors du réveil, l'intensité de l'incorporation du $^{32}P$ dans les acides nucléiques et les phospholipides s'élève, tout en restant inférieure à l'intensité observée chez les animaux normaux réveillés et chez les animaux soumis au sommeil narcotique. Le réveil des hibernants s'accompagne d'un brusque passage de l'état d'inhibition diffuse à l'état d'activité fonctionnelle du cerveau. Il est probable que le réveil forcé entraîne une « collision » de ces deux processus avec développement de l'inhibition transliminaire.

L'utilisation du phosphore marqué a permis d'établir que la pénétration du phosphore du sang dans les tissus, dans le tissu nerveux en particulier, est ralentie, ce qui peut être la cause de la diminution de l'intensité de renouvellement durant l'hibernation (figures 18 et 19).

Dans les états d'inhibition de l'activité nerveuse, qu'il s'agisse du sommeil narcotique ou de l'hibernation, les processus de dégradation sont ralentis, la vitesse du renouvellement du glycogène, de l'acide ribonucléique et des phospholipides est diminuée, la quantité d'ammoniaque est abaissée le taux du glycogène et de l'ATP augmente ; on voit s'établir les conditions plus favorables pour les processus de synthèse, ce qui assure la restauration de la capacité de travail du cerveau.

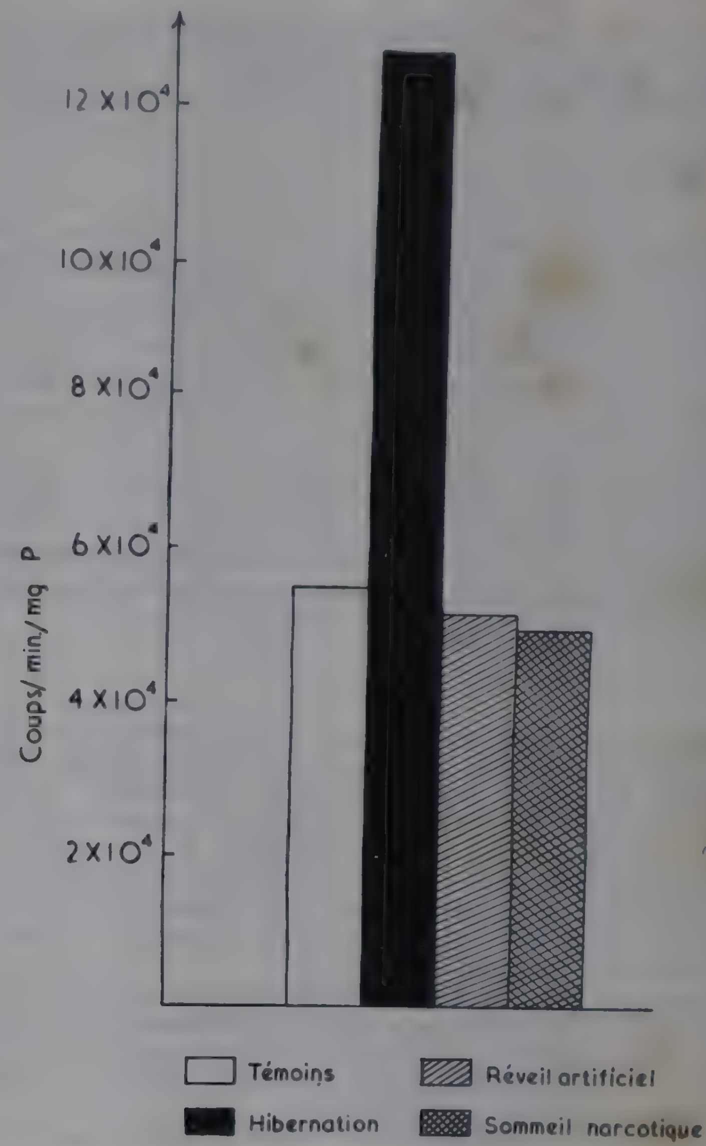


FIG. 18. — Activité spécifique de la fraction acido-soluble du sérum sanguin de spermophiles tués 4 heures après injection de $^{32}P$.

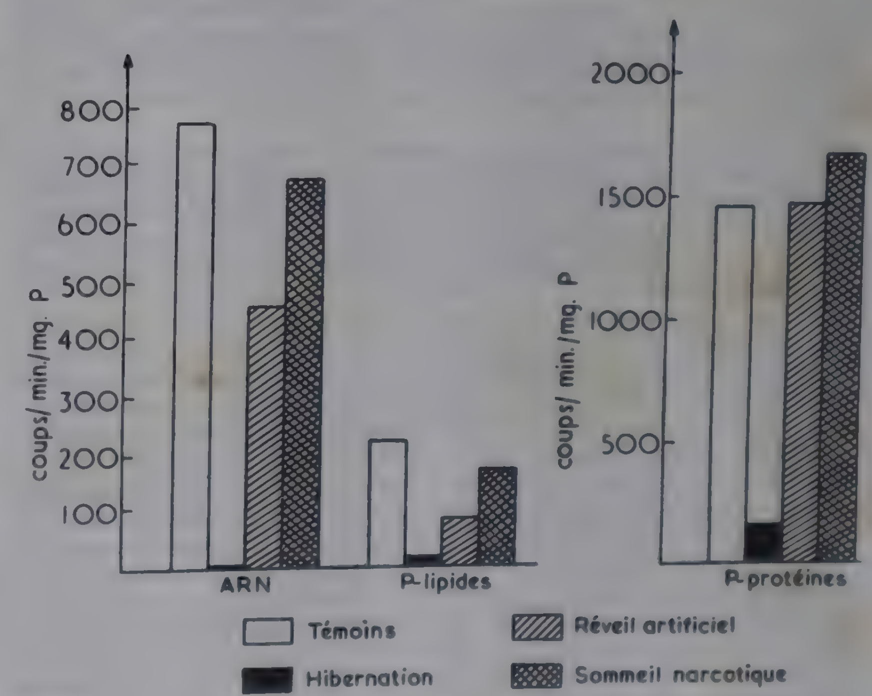


FIG. 19. — Activité spécifique relative de la fraction acido-soluble du cerveau et de la moelle épinière des spermophiles tués 4 heures après injection de $^{32}P$.

Activité spécifique relative = (activité spécifique de la fraction acido-soluble du cerveau/activité spécifique de la fraction acido-soluble du sérum) × 100.

Tels sont les résultats essentiels de plusieurs recherches récentes, dont les nôtres, concernant la biochimie du cerveau et son métabolisme dans différents états fonctionnels et dans les diverses conditions du milieu intérieur et extérieur en particulier.

L'étendue limitée de ce rapport ne m'a pas permis d'exposer tous les aspects de la biochimie du cerveau. Plusieurs études portant sur des problèmes très intéressants du métabolisme du cerveau, par exemple, les problèmes du métabolisme de l'énergie, des phosphorylations oxydatives, n'ont pas été effleurés dans mon rapport.

Cependant les données que je viens de présenter représentent un progrès sensible dans le domaine des recherches sur la constitution chimique des parties du système nerveux qui diffèrent au point de vue fonctionnel, des recherches sur les processus métaboliques, surtout ceux du métabolisme glucidique dans le cerveau, des problèmes de la biochimie fonctionnelle du cerveau, de la biochimie des processus d'excitation et d'inhibition qui sont les processus physiologiques essentiels du système nerveux. Ces recherches ont montré que les états d'excitation et d'inhibition s'accompagnent de modifications différentes du métabolisme du cerveau qui sont en somme opposées.

Il reste beaucoup de problèmes inexplorés; un vaste champ de recherches à effectuer s'offre à la biochimie fonctionnelle du cerveau dont le but final est d'élucider tous les fondements biochimiques de l'activité spécifique de diverses parties du cerveau et de pénétrer au fond du métabolisme cérébral à un degré tel qu'il soit possible de les régler.

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Одной из наиболее интересных и увлекательных и, вместе с тем, одной из наиболее сложных проблем биохимии является проблема биохимии головного мозга, проблема химических основ его деятельности. Исследование химического состава мозга и особенностей обмена веществ в нем представляет большие трудности благодаря многообразию клеточных и проводниковых структур, сложнейшему распределению серого и белого вещества, чрезвычайному богатству ткани мозга лабильными соединениями.

Однако в настоящее время уже имеется большой экспериментальный материал как в области биохимической статики и динамики мозга, так и в области функциональной биохимии мозга, который позволяет в известной мере проникнуть в специфику нервной деятельности.

В настоящем докладе я хочу изложить результаты исследований по биохимии головного мозга, ведущихся ряд лет в Институте биохимии Академии наук Украинской ССР, равно как результаты новейших исследований в этой области других ученых.

Основным положением современной физиологии является представление о целостности и пластичности животного организма, неразрывно связанного с изменяющейся средой, влияющей на его состояние, на развитие и изменение его функциональных свойств и форм как в онтогенезе, так и в филогенезе.

У высших животных, и особенно у человека, целостность организма и его взаимодействия с внешней и внутренней средой регулируется и организуется, как особенно наглядно показали исследования Сеченова и Павлова, центральной нервной системой, которая коррелирует все функции животного организма.

Перед современной биохимией животных и человека, которая должна быть функциональной биохимией, в качестве основной задачи стоит изучение обмена веществ целостного организма при различных функциональных состояниях и воздействиях внешней среды, выяснение связи между специфическими функциями органов и систем и особенностями их обмена, в первую очередь нервной системы, и вскрытие механизма регуляции нервной системой процессов обмена веществ.

В наших исследованиях по биохимии головного мозга мы ставили себе задачей выяснить, существуют ли отличия в химическом строении отдельных участков головного мозга, выполняющих различные функции, и в обмене веществ в них, изменяются ли, и как, процессы обмена веществ в отдельных частях головного мозга при различном функциональном состоянии их и под влиянием тех или иных факторов.

### *Методы работы с мозгом*

Для изучения связи между функциональными изменениями и обменом веществ в головном мозгу исследователи прибегали как к опытам in vivo, так и к опытам in vitro.

Опыты на срезах и мозговых кашицах в функционально-биохимических исследованиях могут иметь только подсобнео значение, так как оыты in vitro могут только вскрыть механизм реакций обмена веществ, тогда как опыты in vivo — физиологическое их значение и роль в функции.

Получение срезов мозговой ткани, которая характеризуется быстро протекающим обменом, исключительной чувствительностью к физиологическим воздействиям, может приводить к распаду целого ряда соединений, имеющих первостепенное значение для мозга, в частности — к распаду фосфокреатина, АТФ, кодегидразы. Далее, раздражение нервных элементов, не имеющих естественной связи с другими участками мозга, может дать иную картину, чем та, которая имеется в целом мозге; в неповрежденном целом мозге явления могут протекать иначе, чем в срезах. Это подтверждается результатами некоторых исследований, показавших, что один и тот же агент может вызвать в опытах in vitro эффект другой, или даже противоположный тому, который наблюдается in vivo. Так, например, под влиянием некоторых барбитуратов содержание фосфокреатина в мозгу в опытах in vivo повышается (1, 2), а in vitro понижаетс (3); содержание неорганических фосфатов изменяется также в противоположных направлениях. Правда, в некоторых случаях определенные воздействия дают одинаковый эффект и в опытах in vivo, и в опытах на срезах; так, например, наркотики тормозят дыхание и срезов мозга (4) и неповрежденного мозга животных и человека (5). Поэтому для получения ряда предварительных данных можно, с учетом возможных погрешностей и с определенными оговорками, пользоваться и при изучении проблем функциональной биохимии мозга опытами in vitro.

С другой стороны, хотя опыты in vivo, опыты на целых животных и связаны с целым рядом методических трудностей, только они могут дать окончательный ответ о связи между определенным функциональным изменением и определенными процессами обмена веществ в мозгу.

При изучении состава и обмена веществ в мозгу в момент определенного функционального состояния, особенно обмена лабильных фосфорных соединений, очень большое значение имеет способ умерщвления животного и фиксирования процессов обмена в мозгу.

Для этого Kerr (6) применил в 1935 г. замораживание мозга жидким воздухом: он анестезировал животных и затем у больших животных, при искусственном дыхании, вскрывал черепную крышку и выливал жидкий воздух на полушария, а у маленьких животных черепной крышки не вскрывал, и после этого извлекал мозг. При такой постановке опытов нужно иметь в виду, что анестезия сама по себе может вызвать изменения в обмене веществ.

Чтобы избежать этого, можно сперва быстро отсечь голову животного, извлечь полушария мозга и заморозить их в жидком воздухе, или быстро отсеченную голову бросить в

жидкий воздух и затем уже, после замораживания, извлечь мозг из черепной коробки. Отсечение головы само вызывает резкое раздражение мозга с соответствующим распадом лабильных соединений. Чтобы избежать этого, можно использовать введение животному перед декапитацией гексенала (1 мг 10% раствора), вызывающее мгновенную смерть. Зависимость результатов определения АТФ и креатинфосфата от способа работы с мозгом (замораживание мозга in situ, замораживание после декапитации, перерезывание спинного мозга, наркоз) особенно ясно видна из работы Фердман и Дворниковой (7).

Возможно еще (8, 9) погружение в жидкий воздух целых животных (крыс, мышей). Однако попадание в жидкий воздух целого животного, несомненно, сильно, хотя и кратковременно, раздражает кожные анализаторы, и раздражения из них успевают подействовать на мозг раньше, чем он замерзнет, и могут вызвать изменения в обмене веществ.

В течение последних двух десятилетий для изучения обмена веществ в нервной ткани широко используются радиоактивные изотопы.

Применение радиоактивных изотопов дало, прежде всего, возможность установить, что многие из составных химических веществ головного мозга, о которых раньше думали, что они в мозгу взрослых животных не подвергаются изменениям, в действительности непрерывно расщепляются и синтезируются.

При изучении процессов обмена в головном мозгу наиболее широко используется радиоактивный фосфор ($^{32}P$) как потому, что фосфорным соединениям принадлежит наиболее важная роль в обмене энергии нервной ткани, так и потому, что фосфорные соединения в первую очередь подвергаются превращениям.

### *Белковые вещества*

Важная роль в функции центральной нервной системы, несомненно, принадлежит белкам; на это указывал уже Данилевский (10) в 1891 году. Однако в деле изучения белковых веществ мозга мы пока почти не продвинулись вперед по сравнению с исследованиями Ewald (11), Halliburton (12) и Данилевского.

Трудность изучения белковых веществ мозга связана с его богатством липоидами, с тем, что каждая попытка отделить липоиды от белков с помощью органических растворителей ведет к денатурации белковых веществ.

Как показали исследования ряда авторов (13), наибольшее количество белковых веществ содержится в коре больших полушарий головного мозга; дальше идет белое вещество головного мозга, затем спинной мозг и меньше всего белков содержится в периферических нервах. Изучение нами (14) различных участков серого вещества центральной нервной системы показало, что филогенетически наиболее молодой и функционально наиболее сложный участок серого вещества, а именно серое вещество больших полушарий головного мозга, содержит наибольшее количество белков; меньше их в сером веществе коры мозжечка и в сером веществе подкорковых узлов и еще меньше в сером веществе спинного мозга.

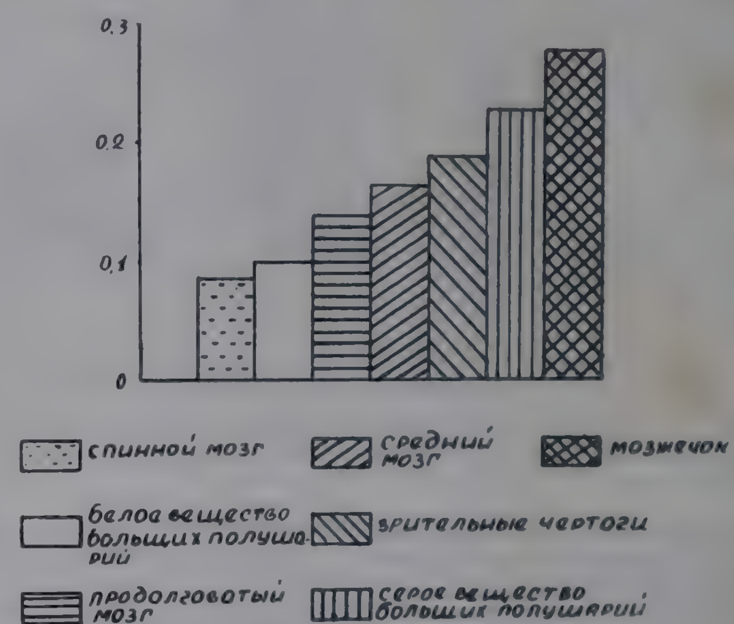
Серое и белое вещество головного мозга, как показали исследования Палладина (15), отличаются друг от друга не только общим количеством содержащихся в них белков, но и качеством белковых веществ, т.е. соотношением отдельных белковых фракций : в сером веществе больше водорастворимых белков и меньше нерастворимого белкового остатка, чем в белом веществе.

В последнее время для изучения обмена белков стали пользоваться методом меченых атомов.

Так, Friedberg, Tarver и Greenberg (16) изучали внедрение метионина, меченого радиосерой ($^{35}S$), в белки различных органов крыс и нашли, что при внутривенном введении метионина мозг по интенсивности внедрения радиосеры, иначе говоря, по скорости обновления белков, далеко уступает другим органам: при введении метионина интрацистериально (когда минуется барьер мозг-кровь) внедрение в мозг происходит интенсивнее, чем в другие органы и ткани. Авторы нашли также, что ход выключения меченого метионина из белков различных тканей хорошо соответствует ходу включения этой аминокислоты, и считают, что о скорости обновления белков правильнее судить по кривой выключения.

Gaitonde и Richter (17), считая, что показателем скорости синтеза белка может служить отношение удельной активности серы белков к удельной активности кислоторастворимой серы, пришли к выводу, что скорость синтеза белка в мозгу сравнительно высока (выше, чем, например, в печени).

Желая определить скорость обновления белков в различных отделах центральной нервной системы, Палладин и Вертаймер (18) изучали интенсивность включения метионина, содержащего меченую серу ($^{35}S$), в белки различных отделов мозга кошек. Исследования показали, что наибольшей скоростью обновления белков обладает серое вещество больших полушарий головного мозга и мозжечок, т.е. функционально наиболее сложные и филогенеически наиболее молодые отделы центральной нервной системы; наименьшей скоростью обновления белков обладает спинной мозг, т.е. функционально наименее сложный и филогентетически наиболее древний отдел центральной нервной системы. К спинному мозгу по интенсивности обновления белков приближается белое вещество больших полушарий. Другие отделы занимают среднее положение (рис. 1).




Фиг. 1. — Относительная удельная активность серы белка.

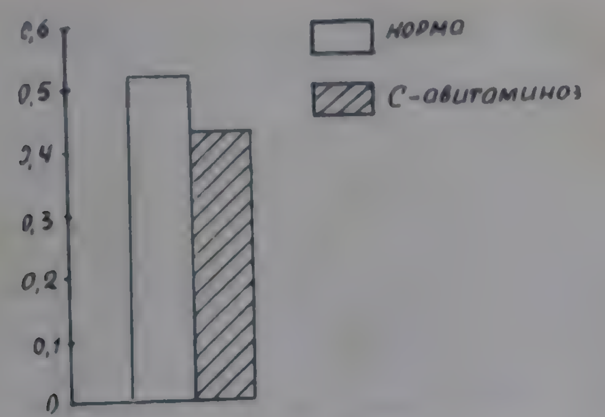
Это подтверждают наблюдения Cohn, Gaitonde и Richter (19), изучавших с помощью ауторадиографического метода внедрение $^{35}S$ после интраперитониального или интрацистериального введения $^{35}S$-метионина в белки головного мозга и нашедших, что внедрение идет более интенсивно в серое вещество, чем в белое вещество мозга.

Таким образом, если абсолютную скорость обновления белков мозга определить с достоверностью еще не удалось, то относительная скорость обновления белков в различных отделах мозга уже в известной мере выяснена.

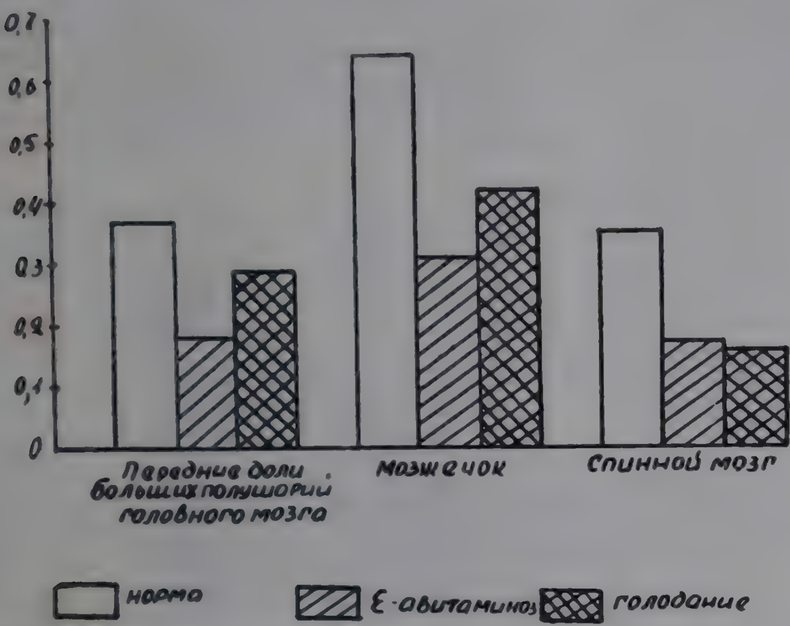
Мы изучали (18) также с посощью меченого метионина интенсивность обновления белков при двух авитаминозах — С-авитаминозе и Е-авитаминозе. При С-авитаминозе наблюдается небольшое снижение интенсивности обновления белков головного мозга морских свинок (рис. 2). Е-авитаминоз значительно сильнее влияет на обмен белков в головном мозгу. При Е-авитаминозе скорость обновления белков в больших полушариях головного мозга, в мозжечке и спинном мозгу кроликов снижается, в среднем, на 50%. Одно голодание вызывает у кроликов значительно меньшее снижение интенсивности обновления белков в больших полушариях и в мозжечке (рис. 3).

Важная роль в мозгу принадлежит нуклеопротеидам (рибонуклеопротеидам и дезоксирибонуклеопротеидам).

Интересные данные о нуклеопротеидах получены Hyden (20) при исследовании замороженных срезов нервных клеток с




Фиг. 2. — Относительная удельная активность белков головного мозга морских свинок при С-авитаминозе.




Фиг. 3. — Относительная удельная активность, белков разных отделов центральной нервной системы кроликов при Е-авитаминозе и голодании.

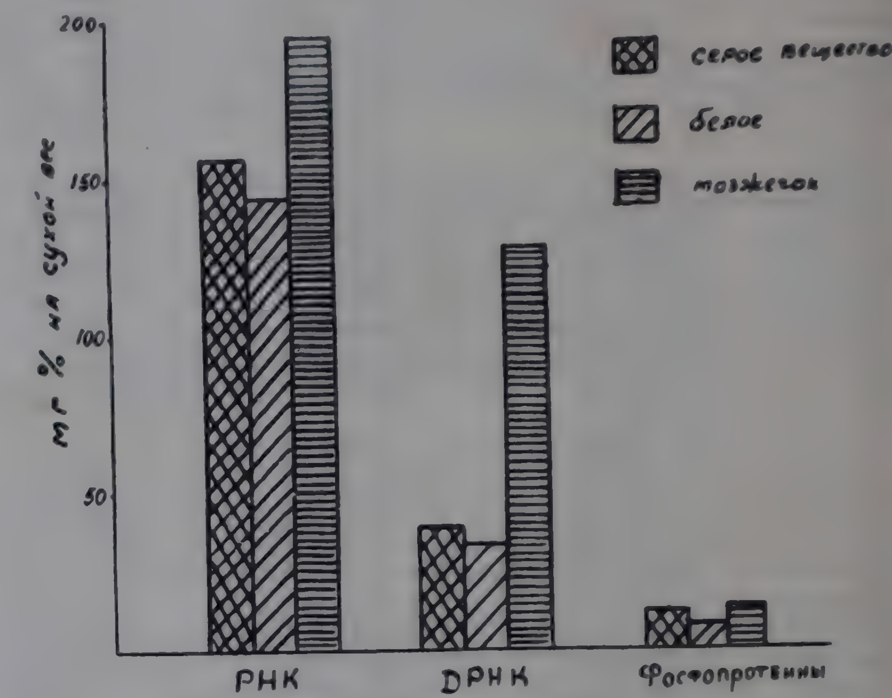
помощью рентген-микро-радиографического метода. Определяя содержание липоидов, пентозонуклеопротеидов и белков, он нашел, что с возрастом состав нервных клеток меняется и в них уменьшается содержание нуклеопротеидов, место которых занимают липопротеиды.

При усиленной деятельности нейрона (при адэкватном раздражении) содержание нуклеопротеидов уменьшается, в то время как белковый остаток не меняется. Таким образом, изменения в веществе нервной клетки бывают двух типов: одни, наблюдаемые с возрастом, развиваются медленно; другие происходят постоянно и заключаются в распаде и восстановлении нуклеопротеидов; их можно рассматривать, как химическую основу функции нейрона.

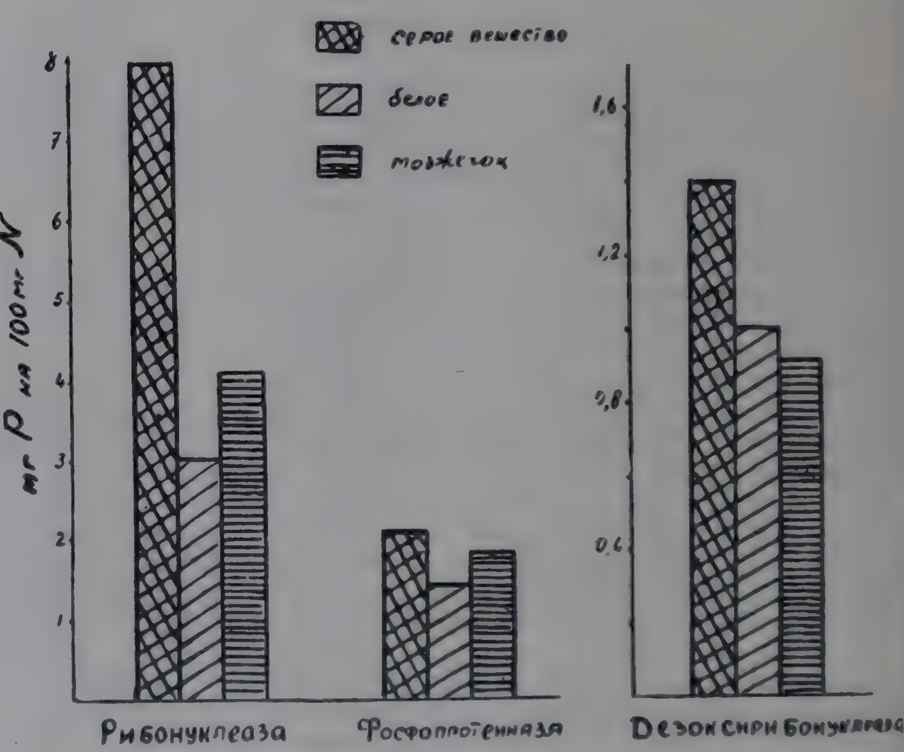
Изучая возрастные изменения структурных белков мозга белых крыс, Буланкин (21) с сотрудниками также нашел, что с возрастом идет вытеснение нуклеиновых кислот соединениями липоидного типа. Такие же возрастные изменения констатированы и в целой ткани мозга. Одновременно нуклеопротеиды обогащаются белком.

Литературные данные говорят о наличии определенного соответствия между интенсивностью синтеза белков и содержанием нуклеиновых кислот в тканях. Значительное количество нуклеиновых кислот в нервной ткани позволяет предположить, что они играют важную роль в деятельности нервной системы. Это и заставляет изучать обмен нуклеиновых кислот в мозговой ткани и его связь с физиологической деятельностью мозга.

Сквирская и Силич (22), изучая содержание нуклеиновых кислот в различных отделах центральной нервной системы, нашли, что кора головного мозга и белое вещество как по общему содержанию нуклеиновых кислот, так и по содержанию отдельных фракций близки друг к другу, при некотором преобладании их содержания в коре. В мозжечке содержание нуклеиновых кислот, особенно дезоксирибонуклеиновой кислоты, значительно выше, чем в сером и, тем более, в белом веществе больших полушарий головного мозга (рис. 4 и 5).




Фиг. 4. — Содержание некоторых фосфорных фракций в сером и белом веществе больших полушарий мозга и в мозжечке.




Фиг. 5. — Активность ферментов в различных отделах головного мозга кроликов.

С помощью ранее разработанного Палладиным, Рабша и Штутман метода (23) выделения ядер серого вещества головного мозга, который дал возможность установить, что нуклеопротеид ядер является, в основном, дезоксирибонуклеопротеидом и что в ядрах коры содержится 20-30% рибонуклеиновой кислоты и 70-80% ДРНК, было изучено (22) содержание нуклеиновых кислот в ядрах мозжечка: оказалось, что в ядрах мозжечка количество рибонуклеиновой кислоты составляет 12-13% всех нуклеиновых кислот, а дезоксирибонуклеиновой кислоты 87-88%, т.е. что в ядрах мозжечка рибонуклеиновой кислоты меньше, чем в ядрах коры больших полушарий. Эти наблюдения заставляют думать о наличии определенной зависимости между составом клеточного ядра и функцией клеток.

Судя по активности деполимеризующих ферментов — рибонуклеазы и дезоксирибонуклеазы, можно думат, что обмен нуклеиновых кислот протекает более интенсивно в сером веществе, чем в белом: активность рибонуклеазы и дезоксирибонуклеазы наиболее высока в сером веществе; она значительно ниже в белом веществе; мозжечок знаимает среднее положение.

*Активность рибонуклеазы, выраженная в мг P на 100 мг и, отщепленного за 1 час инкубации, и дезоксирибонуклеазы, выраженная как разница между начальной и конечной относительными вязкостями.*

| | Рибонуклеаза | Дезоксирибонуклеаза |
|---|---|---|
| Серое вещество больших полушарий | 7,92 | 1,40 |
| Белое вещество больших полушарий | 3,03 | 1,00 |
| Мозжечок | 4,14 | 0,85 |

В последнее время уделяется много внимания методам определения нуклеиновых кислот в тканях и, в частности, в мозгу, поскольку при фракционировании рибонуклеопротеидов по Шмидту и Тангаузеру они оказываются загрязненными другими веществами (24, 25, 26).

Исследования с помощью радиофосфора обновления фосфора нуклеиновых кислот как в целом мозгу (22, 27, 28), так и в срезах мозга (29) показали, что фосфор дезоксирибонуклеиновой кислоты обменивается чрезвычайно медленно, а рибонуклеиновой — несколько быстрее, но все же медленнее, чем фосфопротеиды. Изучая скорость обмена фосфора нуклеиновых кислот в разных отделах мозга, мы нашли (22), что скорость обновления фосфора рибонуклеиновой кислоты в сером веществе мозга кроликов ниже, чем в белом веществе и в мозжечке.

Аналогичные данные получил Крепс (28): он нашел, что у кроликов скорость обновления рибонуклеиновой кислоты в коре ниже, чем в мозжечке, промежуточном, среднем и в продолговатом мозге; у собак в больших полушариях наблюдается, наоборот, более высокая скорость обновления фосфора рибонуклеиновой кислоты по сравнению с другими отделами, в чем находит свое отражение более высокое функциональное развитие больших полушарий у собак.

Различные зоны коры больших полушарий мозга собаки, соответствующие разным корковым анализаторам, обладают различной интенсивностью обмена фосфорных соединений: наибольшей скоростью обновления рибонуклеиновой кислоты (и фосфолипидов) обладает зона двигательного анализатора.

Большая скорость обновления рибонуклеиновой кислоты в белом веществе больших полушарий (а также спинного мозга) указывает на то, что белое вещество отнюдь не является инертной в отношении обмена частью мозговой ткани.

В обмене белковых веществ следует искать ключ к разгадке функциональных особенностей ткани головного мозга. С этой точки зрения несомненный интерес представляет фракция белков, носящая название фосфопротеинов и встречающаяся в ряде тканей животного организма, в том числе и в нервной ткани. О выполнении фосфопротеинами каких-то важных функций в головном мозгу говорит чрезвычайно быстрая обмениваемость их фосфора, установленная опытами с применением меченого фосфора $^{32}P$ (30, 31, 32). Скорость обмена фосфора фосфопротеинов далеко превосходит скорость обмена нуклеиновых кислот и фосфолипидов.

Энгельгардт (33) на срезах из серого вещества мозга крых подтвердил, что фосфопротеины серого вещества мозга обмениваются со скоростью, превышающей скорость обмена других фосфорных соединений, в частности нуклеиновых кислот, и нашел, что обмен фосфопротеинов зависит от протекания окислительных процессов и связан, в первую очередь, с механизмом окислительного фосфорилирования.

Белковые вещества в мозгу находятся в связи не только с нуклеиновыми кислотами, но образуют комплексы с целым рядом других веществ — липоидами, холестерином, углеводами (гликогеном).

Вопрос о наличии липопротеинов в мозгу изучен еще недостаточно, хотя их присутствие в нервной ткани вполне возможно.

Folch (34) выделил из мозга протеолипоиды, растворимые в органических растворителях (прежде всего в содержащем спирт хлороформе) и отличающиеся от липопротеидов особенно высоким содержанием липоидов. Протеолипоиды являются чрезвычайно лабильными веществами. Протеолипоиды мозга человека и быка (35) по содержанию общего азота, фосфора и по аминокислотному составу весьма сходны.

*Углеводы*

В нервной ткани нет больших запасов углеводов, хотя они играют в ней важную роль, являясь основным источником энергии. Содержание гликогена в головном мозгу колеблется в пределах от 70 до 130% (36).

После открытия фосфорилазы, обусловливающей расщепление гликогена в мышцах (а также в печени), стали вообще отрицать существование амилазы в тканях животного организма: были забыты и старые данные (37, 38, 39) о наличии амилазы в ткани головного мозга. Однако выполненные в нашем институте исследования Рашба (40) показали, что в головном мозгу амилаза содержится, и притом очень активная; она связана с белками головного мозга и освобождается при автолизе. Под влиянием амилазы и происходит, в основном, расщепление гликогена в головном мозгу. Роль фосфорилазы в головном мозгу связана, главным образом, с синтезом полисахаридов.

Cori (41) высказали предположение, что гликоген в мозгу образуется под влиянием фосфорилазы (синтезирующей неразветвленный полисахарид типа амилозы) и другого дополнительного фермента, вызывающего превращение амилозы в разветвленный полисахарид (гликоген). В нашем институте Хайкина и Гончарова (42) выделили оба эти фермента (фосфорилазу и изомеразу) из ткани головного мозга, разделив их путем фракционирования сернокислым аммонием.

Изучая ферменты, вызывающие распад и синтез гликогена, мы (43) исследовали также его содержание в разных отделах мозга и смогли обнаружить гликоген в коре больших полушарий и в мозжечке здоровых животных, а также нашли, что он подвергается непрерывным превращениям.

Несостоятельность мнения о неизменяемости содержания гликогена в мозгу особенно наглядно видна из результатов изучения углеводного обмена головного мозга при различных его функциональных состояниях: при судорогах, напр., сильно повышается активность амилазы и уменьшается содержание гликогена (44).

О быстро протекающем обмене гликогена говорит и то, что скорость обновления меченого углерода гликогена мозга равна по данным Прохоровой (45), или даже превышает, скорость обновления гликогена печени. Такой же вывод следует сделать из исследований изменений в содержании гликогена в коре больших полушарий, среднем и продолговатом мозгу и мозжечке мышей при различных условиях (46).

Обсуждение вопроса о том, может ли гликолиз в мозгу итти без участия фосфора, имеет только исторический интерес; теперь ясно, что гликолиз, как реакция, доставляющая энергию, может итти только при участии фосфора. К тому же, на мозговых экстрактах были обнаружены такие же промежуточные продукты гликолиза, как и в мышцах. Были обнаружены аналогичные ферменты углеводного обмена.

Так, мы подтвердили данные Ochoa (47) и др. о наличии в мозгу гексокиназы, найдя ее и в сером и в белом веществе (48); у молодых животных она более активна, чем у взрослых (49). Мы изучили свойства и роль и других находящихся в мозгу ферментов, как-то фосфоглюкомутазы (50), альдолазы (51) и аденозинтрифосфатазы (52); свойства последней изучал также Gore (53). Гликолиз протекает с различной интенсивностью в различных отделах мозга; об этом говорят результаты наших исследований ферментов углеводного обмена, показавшие, что гексокиназа, альдолаза, фосфорилаза и аденозинтрифосфатаза обладают наибольшей активностью в коре больших полушарий и в мозжечке.

При окислительном обмене глюкозы сперва образуется пировиноградная кислота, дальнейшее окисление которой происходит и в нервной ткани, несомненно, по пути лимоннокислого цикла.

*Липоиды*

В химическом строении нервной ткани большую роль играют липоиды. Почти половина сухого вещества головного мозга приходится на долю липоидов; в спинном мозгу их еще больше. В головном мозгу липоидов больше в белом веществе, чем в сером.

Липоиды головного мозга состоят, в основном из холестерина, глицерин-фосфатидов, сфингомиэлина и цереброзидов.

Строение глицерин-фосфатидов еще недостаточно изучено. Кроме давно известных глицерин-фосфатидов лецитина и кефалина, в нервной ткани содержится еще серин-кефалин, полученный Folch (54) и ацетальфосфатиды Feulgen (55).

Ацеталь-фосфатиды были выделены из мозга Thannhauser и сотр. (56). В мозгу, в основном, содержатся только ацетальфосфатиды, содержащие коламин.

Изолированный Folch (57) из мозга очень богатый углеводами липоид, названный страндином, по мнению Klenk (58), является ганглиозидом.

С целью более глубокого изучения стеринов головного мозга в нашем институте Поляковой (59) было предпринято исследование состава неомыляемой фракции головного мозга животных, а также белого и серого вещества больших полушарий мозга человека, причем для разделения составных частей был использован метод адсорбционной хроматографии. Исследования мозга человека показали, что стеринов в неомыляемой фракции белого вещества больших полушарий содержится 93%, а в неомыляемой фракции серого вещества — 85%, причем в обоих случаях главную массу стеринов составляет холестерин; серое вещество содержит 7-оксихолестерин, которого нет в белом веществе.

Таким образом, функционально различные отделы головного мозга отличаются друг от друга не только по количеству стеринов, но и по качеству их. Опыты in vitro со срезами мозга показали, что мозг крысы может синтезировать фосфолипиды (6). Что же касается синтеза холестерина, то синтез его идет только в срезах мозга новорожденных крыс, а срезы мозга взрослых крыс совсем не синтезируют холестерин (61). Синтез холестерина наблюдали и в опытах in vivo, причем по мере роста крыс синтез холестерина в их мозгу понижается (62).

В последнее время появился ряд исследований, посвященных выделению различных липоидов из разных отделов мозга и изучению, с помощью радиофосфора, скорости внедрения фосфора в различные липоиды (63).

Центральная нервная система не является однородной тканью. Кроме многих типов нейронов с различными обменными процессами, в ней имеется также в большом количестве несколько видов глии и разные другие элементы. Все это делает необходимым проведение более тонких исследований ферментных систем и обменных процессов в индивидуальных клетках и отдельных участках клеток. В этом отношении большую ценность представляют комбинированные биохимические и морфологические исследования Flechner (64), Bodian (65) и Pope (66). К таким исследованиям относятся и недавние исследования Aboad и сотр. (67), в которых они, разделив серое вещество головного мозга и белое вещество спинного мозга на четыре фракции: ядерную, метахондрий, жидкого слоя, липидную, — изучали как содержание в них отдельных веществ (нуклеиновых кислот и ряда фосфорсодержащих веществ), так и ферментную активность (окислительных, фосфорилирующих и ряда гликолитических ферментов).

*Сравнительно-биохимические исследования*

Одним из путей изучения функциональной биохимии центральной нервной системы является путь сравнительно-биохимический, т.е. изучение химических процессов в мозгу в онтогенезе и филогенезе.

Биохимическим изучением головного мозга в онтогенезе занимается с 1946 года Крепс с сотр. (68, 69) в Физиологическом институте им. И. П. Павлова в Ленинграде. Он изучал развитие активности ряда ферментных систем мозга, сопоставляя биохимическое развитие с морфологическим развитием и с функциональным созреванием центральной нервной системы. При изучении карбоангидразы в мозгу позвоночных животных разных классов в онтогенезе было установлено, что у низших позвоночных богаче карбоангидразой стволовая часть мозга, тогда как у высших на первое место постепенно выдвигается кора больших полушарий (70, 71).

Развитие карбоангидразы мозга в онтогенезе является характерным для каждого вида животных, отражая особенности эмбриогенеза и хорошо коррелируя с функциональным развитием нервной системы (72). Можно думат, что карбоангидраза имеет определенное физиологическое значение в мозгу, вероятнее всего связанное с поддержанием нормального щелочно-кислотного равновесия в ткани мозга.

В эволюционном ряду позвоночных животных при переходе от низших его представителей к высшим нарастает интенсивность дыхания и снижается интенсивность анаэробного гликолиза (73). В соответствии с этим в процессе эволюции позвоночных животных происходит увеличение активности цитохромоксидаз и всей цитохромной системы ткани головного мозга (74).

У млекопитающих и у птиц активность ферментных систем окисления и фосфорилирования во взрослом состоянии максимальна в коре больших полушарий, значительно превосходя стволовую часть мозга и, тем более, спинной мозг. Высокой активностью, часто не уступающей коре, отличается мозжечок (75, 76). Эти результаты подтверждают вышеприведенные наши данные о наибольшей активности ферментов углеводного обмена в коре больших полушарий.

Изменение активности холинэстеразы в головном и спинном мозгу молодых крыс в процессе развития изучал Bayliss (77).

Крепс (28) показал, что обновление фосфопротеинов, рибонуклеиновой кислоты и фосфолипидов в разных отделах центральной нервной системы животных происходит с различной скоростью, причем имеется прямая зависимость между уровнем функционального развития и интенсивностью фосфорного обмена в разных отделах мозга.

Bieth (78, 79) с сотрудниками провел сравнительные исследования состава мозга взрослых животных некоторых классов позвоночных, а также изучил кислоторастворимые фосфорные соединения мозга крысы в процессе его развития (от 3 дней до 1 года).

Сквирская и Силич (22), изучая обмен нуклеиновых кислот и фосфопротеинов в головном мозгу кроликов в различные периоды эмбрионального и постэмбрионального развития, нашли, что содержание обеих нуклеиновых кислот в мозгу, очень высокое на ранних стадиях эмбрионального развития, постепенно снижается с возрастом эмбриона. После рождения темп этого снижения падает и к месячному возрасту доходит до уровня, близкого к содержанию в мозгу взрослых животных.

Содержание фосфопротеинов также выше на ранних стадиях развития; оно несколько снижается к 9-дневному возрасту, а к месячному достигает уровня, характерного для взрослого животного.

*Содержание нуклеиновых кислот и фосфопротеинов в мозгу эмбрионов кроликов и кроликов различного возраста (в мг% на сухое вещество).*

| Возраст | Фосфор | | |
|---|---|---|---|
| | РНК | ДРНК | ФП |
| Эмбрионы: | | | |
| 16-20 дней | 416 | 368 | 20,8 |
| 26-29 дней | 321 | 140 | 31,0 |
| Новорожденные | 298 | 148 | 21,7 |
| Молодые кролики: | | | |
| 9-10 дней | 236 | 89 | 18,0 |
| 1 мес. | 195 | 48 | 3,7 |
| Взрослые | 166 | 37 | 6,7 |

Изменения активности дезоксирибонуклеазы мозга совпадают с появлением новых функций: так, активность дезоксирибонуклеазы возрастает на 20-й день эмбрионального развития, что, вероятно, связано с усиленной дифференцировкой органов. Второй подъем активности дезоксирибонуклеазы наблюдается на 9-й день постнатального развития, т.е. в период прозревания, иначе говоря — начала зрительной функции.

*Обмен веществ и функция*

Головной мозг характеризуется очень активным обменом веществ. В бодрствующем состоянии температура мозга на 0,5° превышает температуру артериальной крови. Мозг взрослого человека поглощает около четверти общего количества кислорода, потребляемого человеком (80).

Процессы обмена веществ в головном мозгу никогда не прекращаются, так как всегда продолжается его деятельность. В зависимости от степени функциональной деятельности мозга находится и метаболическая активность мозга, которая постоянно меняется. Самые большие изменения мозговой деятельности и обмена веществ в мозгу, наблюдаемые в экспериментальных условиях, — это те, которые обнаруживаются при раздражении и при угнетении. Поэтому влияние этих двух функциональных состояний на обмен веществ в мозгу изучалось особенно часто.

Так, например, исследования показали, что в состоянии пониженной функции (сон или наркоз) богатые энергией фосфорные соединения накапливаются, а в состоянии повышенной деятельности расщепляются, что проявляется в повышении гликолиза и в усиленном образовании молочной кислоты. Olsen и Klein (81) нашли, что при судорогах, вызванных раздражением электрическим током, уменьшается содержание в мозгу кошки глюкозы, гликогена, аденозинтрифосфорной кислоты и фосфокреатина, увеличивается содержание неорганического фосфора и молочной кислоты. Содержание молочной кислоты повышается также шри судорогах, вызванных введением камфоры и стрихнина (82); в подобных опытах обнаружены видовые отличия: содержание АТФ меньше меняется у крыс, чем у кошек.

Особенно быстро происходит при раздражении распад фосфокреатина: содержание АТФ при этом уменьшается в небольшой степени, так как ее запасы восполняются за счет фосфокреатина. Через 15-30 секунд после электрического раздражения содержание фосфокреатина постепенно возвращается к норме (83).

Опыты с действием наркотиков показали высокий уровень фосфокреатина и АТФ, низкое содержание молочной кислоты в мозгу; таким образом, изменения при наркозе в общем обратны изменениям при судорогах. Наркотики угнетают дезаминазу адениловой кислоты (84).

Dawson и Richter в опытах с $^{32}P$ наблюдали торможение синтеза нуклеопротеидов и фосфолипидов в мозгу мышей при нембуталовом наркозе (85). При сне содержание молочной кислоты в мозгу крыс также уменьшается (86), правда, в несколько меньшей степени, чем при наркозе.

McIlwain с сотрудниками применил для изучения in vitro обмена веществ в мозгу при раздражении метод раздражения срезов мозга электрическим током (87); Ayres и McIwain разработали для этого специальную аппаратуру (88). Исследования показали, что при раздражении срезов мозга, так же как и в опытах in vivo усиливается дыхание и увеличивается образование молочной кислоты (89), уменьшается содержание креатинфосфата и увеличивается неорганический фосфат (90), аналогичное действие оказывает 2,4-dinitrophenol.

McIlwain изучал на срезах обмен мозговой ткани в условиях, воспроизводящих тяжелую гипогликемию, при которой наступает падение активности мозга. Истощенная мозговая ткань не отвечала на электрическое раздражение повышением дыхания при добавлении в среду глюкозы, однако не теряла способности увеличивать образование молочной кислоты из глюкозы в ответ на раздражение (91). Исследуя дыхание и гликолиз в срезах мозга человека, кролика и морской свинки при разном содержании глюкозы в среде и влияние на дыхание и гликолиз электрических импульсов, McIlwain (92) нашел, что электрические импульсы усиливали дыхание.

Heald (93) изучал превращения креатин-фосфата в срезах коры при прохождении электрических импульсов и нашел, что при прохождении импульсов креатинфосфат распадается со скоростью 1400 миллимолей/гр/час после латентного периода в 2-3 секунды; распад оканчивается в течение 5 секунд. Распаду креатинфосфата предшествовало слабое падение уровня аденозинтрифосфата, который восстанавливался, когда начинался распад креатинфосфата. Если электрические импульсы прекращались через 7 секунд, креатинфосфат ресинтезировался до начального уровня за 20 секунд.

Ряд исследователей занимался вопросом образования аммиака в головном мозгу при возбуждении и угнетении нервной деятельности. Владимирова (1938) установила (94, 95), что возбуждение центральной нервной системы с помощью камфоры сопровождается увеличением образования аммиака (а также молочной кислоты), а угнетение, вызываемое уретаном, сопровождается уменьшением содержания аммиака в мозговой ткани. Безусловное болевое раздражение (электрический ток) и выработанное на его основе рефлекторное возбуждение также вызывают повышение содержания аммиака в мозгу (96). Обратно, всякое воздействие, приводящее к состоянию торможения, приводит к снижению содержания аммиака в мозгу.

Richter и Dawson (1948) также нашли (97), что содержание аммиака в мозгу возрастает при судорогах, вызванных введением пикротоксина, а также в результате электрического раздражения и при аноксии и уменьшается при нембуталовом наркозе.

Источником образования аммиака в мозгу является не только адениловая система (98), как это имеет место в мышечной ткани. Образование аммиака в мозгу происходит также за счет потери глютамином аминной группы и его превращения при этом в глютаминовую кислоту. Во время восстановительного периода после раздражения происходит устранение аммиака с возрастанием глютамина (99, 100). Таким образом, система глютамин-глютаминовая кислота участвует в регуляции содержания аммиака в мозговой ткани.

*Обмен веществ при возбуждении нервной деятельности*

Занимаясь в течение ряда лет исследованиями (101, 102) обмена веществ в головном мозгу при различном функциональном состоянии (некоторые результаты которых уже были изложены выше), я с моими сотрудниками особое внимание обратил на изучение процессов обмена веществ в мозгу при возбуждении и торможении пернвой деятельности (103, 104, 105, 106).

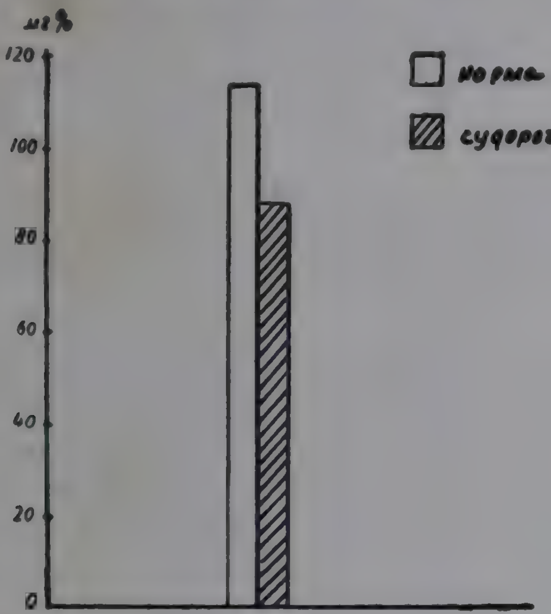
Павлов не раз указывал, что основными процессами, характеризующими высшую нервную деятельность, являются процессы возбуждения и торможения и что расшифровка их в значительной мере зависит от изучения физики и химии нервной системы (107).

При постановке наших исследований над обменом веществ при возбуждении и торможении мы учитывали, что состояние возбуждения и торможения в головном мозгу может быть вызвано в эксперименте различными путями. От того, чем вызывается то или иное состояние нервной системы, могут зависеть и некоторые отличия процессов обмена веществ. Однако, считая, что прежде всего нужно установить основные особенности обмена веществ в головном мозгу при возбуждении и торможении, мы на первом этапе считали возможным воспользоваться фармакологическими средствами, чтобы, установив известные закономерности общего порядка, перейти затем к использованию раздражителей физиологических, к использованию как безусловных, так и условных раздражителей.

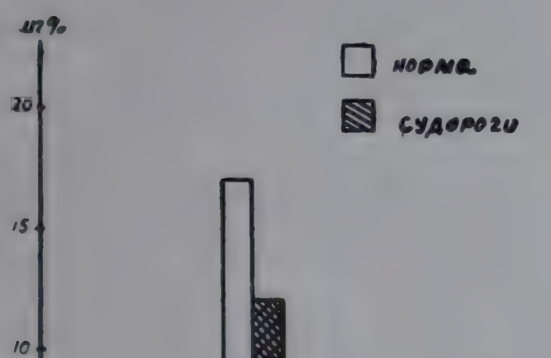
Физиологическими раздражителями мы уже пользовались на самом начальном этапе наших работ в области биохимии головного мозга, когда Городисская (108) изучала влияние естественных раздражений на процессы протеолиза в зрительной зоне коры больших полушарий и установила, что переход зрительных центров в состояние более повышенной деятельности связан с усилением процессов белкового обмена.

Наши исследования были направлены на изучение обмена нуклеиновых кислот, углеводов, аденозинтрифосфорной кислоты, фосфопротеинов и фосфолипидов. Опыты проводились на кроликах, а также на собаках и крысах. Чтобы избежать распада лабильных фосфорных соединений в связи с раздражением в момент декапитации, мы перед декапитацией вводили животному внутривенно 1 мл 10%-го раствора гексенала. Мозг замораживался в жидком воздухе. Состояние возбуждения мы вызывали введением первитина, который, подобно бензедрину, широко применяется в качестве стимулятора нервной системы, и кардиазола, являющегося также практически используемым препаратом.

Прежде всего мы изучали влияние длительного возбуждения (вызванного введением больших доз кардиазола или раздражением электрическим током), вызывавшего судороги. В этом случае в мозгу уменьшается содержание гликогена и аденозинтрифосфорной кислоты (109, 110). Таким образом, наши данные (рис. 6 и 7) подтверждают результаты исследований Olsen и Klein (81) и других авторов (111, 1).



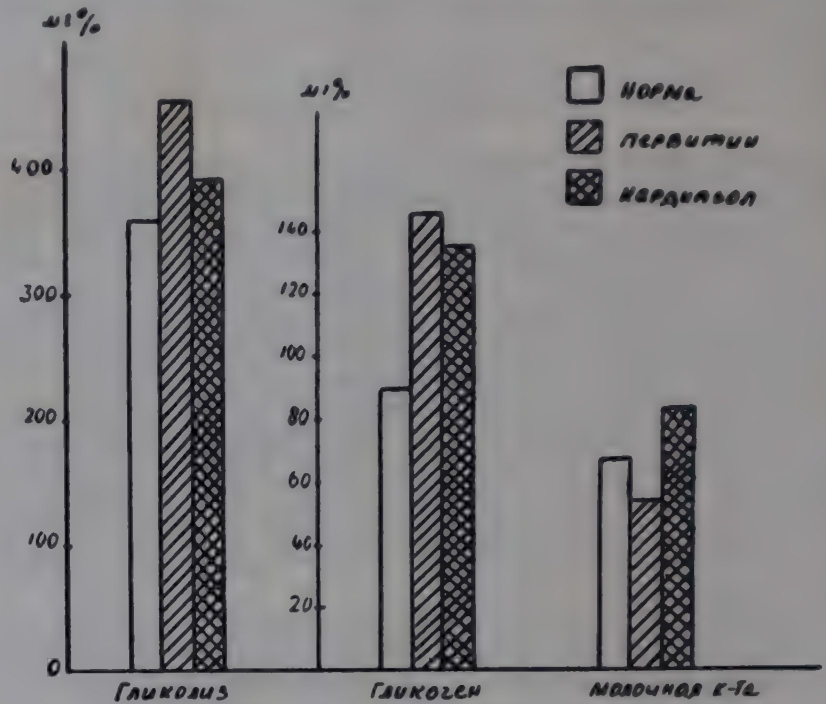

Фиг. 6. — Содержание гликогена в мозгу содак при судорожном состоянии.




Фиг. 7. — Содержание АТФ в мозгу крыс при судорожном состоянии.

В исследовании более физиологического порядка, когда возбуждение вызывалось одноразовым введением первитина (5-7 мг на 1 кг веса) или кардиазола (50-70 мг на 1 кг веса) за 4 часа до умерщвления животного, мы нашли, что различные возбуждающие вещества вызывают неодинаковые изменения в обмене веществ головного мозга (109).

При возбуждении, вызванном первитином, содержание преформированной молочной кислоты оказывается пониженным и по сравнению с нормой, и по сравнению с кардиазоловым возбуждением (112). Содержание АТФ и гликогена при возбуждении с помощью первитина оказывается повышенным, а при кардиазоловом возбуждении содержание АТФ почти не отличается от нормы (рис. 8).




Фиг. 8. — Содержание гликогена, молочной кислоты и интенсивность гликолиза при воздуждении в мозгу кроликов.

При возбуждении, вызванном первитином, содержание рибонуклеиновой кислоты немного увеличивается (113).

Изучение фосфолипидов при первитиновом возбуждении показало (114), что как в общем содержании фосфолипидов, так и в содержании фракций насыщенных и ненасыщенных фосфолипидов в течение 3-х часов после введения первитина заметных изменений не наблюдается.

Однако применение радиофосфора дало возможность установить, что внедрение фосфора в обе фракции фосфолипидов при первитиновом возбуждении происходит иначе, чем в норме; значит, при изменении функционального состояния нервной системы обмен фосфолипидов меняется. Таким образом, наши исследования показали, что различные возбуждающие вещества по-разному влияют на обмен веществ в головном мозгу и что с этим связаны различия в физиологическом эффекте при их применении: при первитине процессы углеводного обмена усиливаются и накапливается биохимически и физиологически активное вещество — АТФ, что и обеспечивает повышение работоспособности нервной системы под влиянием первитина, обладающего стимулирующим действием на нервную деятельность. Кардиазол возбуждает кору мозга, не повышая ее работоспособности.

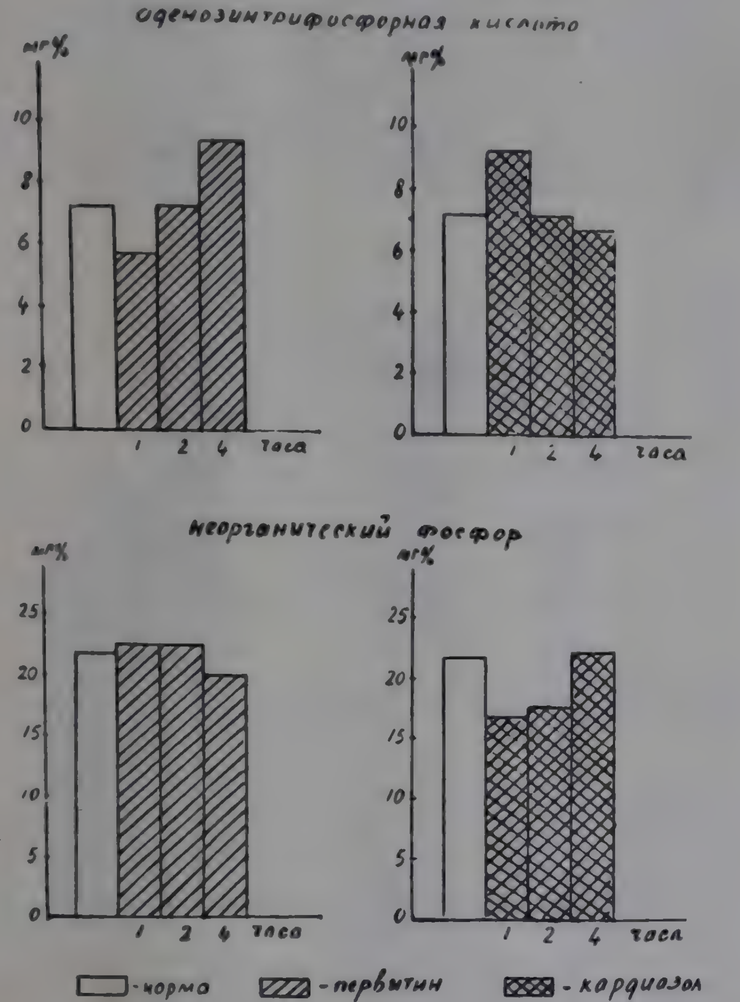
Владимиров (115) изучал влияние возбуждения центральной нервной системы на скорость обновления рибонуклеиновой кислоты и фосфолипидов. Возбуждение вызывалось путем воздействия в течение трех часов (с интервалом отдыха) электрическим током на рецепторы кожи крыс. Исследования показали, что состояние возбуждения головного мозга сопровождается, судя по результатам определения относительной удельной активности фосфора, повышением интенсивности обновления фосфора рибонуклеиновой кислоты и фосфолипидов; при этом интенсивность обмена рибонуклеиновой кислоты увеличивается на 20%, а интенсивность обмена фосфолипидов возрастает в полтора раза.

Принимая во внимание, что характер возбуждения может быть различным как в зависимости от природы возбуждающего вещества, так и продолжительности его воздействия на нервную систему, мы изучили обмен фосфорных соединений в мозгу в различные сроки после введения первитина или кардиазола (через 1, 2 и 4 часа).

Исследования показали (116), что при возбуждении, вызванном первитином, содержание АТФ в головном мозгу через 1 час после введения первитина оказывается понижен-

ным, затем повышается и через 2 часа доходит до нормальных величин; продолжая повышаться дальше, содержание АТФ через 4 часа после введения первитина оказывается значительно повышенным по сравнению с нормой. Таким образом, эти данные подтвердили результаты только что изложенных исследований, в которых содержание АТФ в головном мозгу определялось через 4 часа после введения первитина. Вместе с тем они показали, что в различные периоды возбуждения обмен АТФ протекает неодинаково. Изменения в содержании неорганического фосфора дают обратную картину.

При возбуждении, вызванном кардиазолом, наблюдалась другая картина: АТФ через 1 час после введения кардиазола было повышено, а затем падало и через 2 часа, а особенно через 4, было пониженным по сравнению с нормой. Неорганический фосфор дает обратную картину (рис. 9).



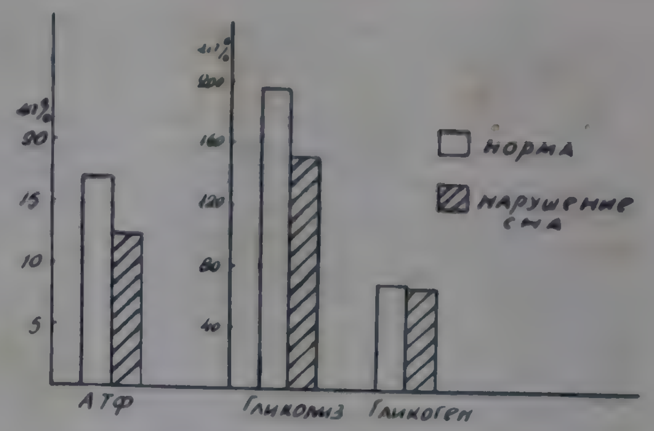

Фиг. 9. — Содержание фосфора аденозинтрифосфорной кислоты и неорганического фосфора в головном мозге кроликов при воздуждении.

Эти опыты еще раз показали, что первитин и кардиазол, обладающие различным физиологическим действием, оказывают различное влияние на обмен АТФ в головном мозгу.

Далее мы провели исследования с хроническим перевозбуждением, которое мы вызывали или длительным раздражением электрическим током, или нарушением физиологического сна. В первом случае крысы, помещенные в специальную электродную клетку, подвергались ежедневному в течение длительного времени (1 - 1,5 месяца) воздействию электрического тока силой в 25-40 вольт. Во втором случае мы сажали крыс на 3 суток во вращающийся барабан, который через каждые 5 минут вращался в течение 30 секунд; в результате крысы в течение 3 суток не могли спать.

Исследования показали (117), что при хронической бессоннице интенсивность гликолиза несколько снижается; количество гликогена почти не меняется; количество аденозинтрифосфорной кислоты уменьшается (рис. 10). При многодневном раздражении электрическим током количество аденозинтрифосфорной кислоты также уменьшается.

Изучение обмена нуклеиновых кислот при длительном возбуждении электрическим током показало (22), что содер-




Фиг. 10. — Обмен углеводов и АТФ при нарушении сна у крыс.

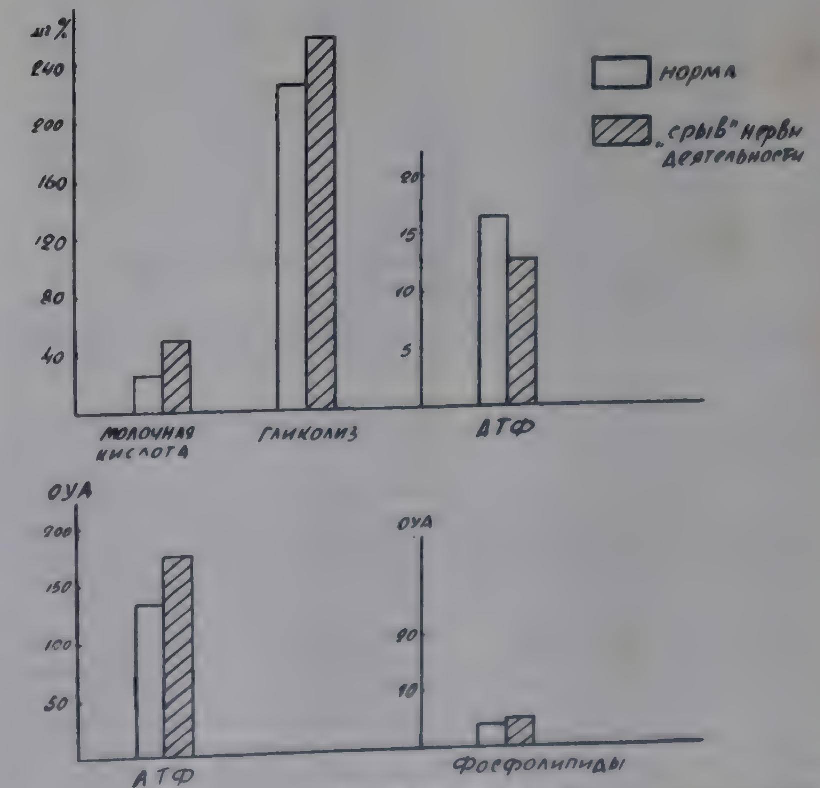
жание нуклеиновых кислот и фосфопротеинов почти не меняется.

Применение меченого фосфора дало возможность установить, что, несмотря на отсутствие изменений в содержании нуклеиновых кислот, их обмен при хроническом возбуждении нервной системы меняется, а именно скорость обновления рибонуклеиновой кислоты снижается.

С целью дальнейшего изучения обмена веществ в головном мозгу при перенапряжении нервных процессов Хайкина и Крачко (118) изучили обмен веществ при « срыве » нервной деятельности, наступающем в результате столкновения двух противоположных раздражений: условного, пищевого (звонок) и безусловного (электрический ток). Выработка условных рефлексов у крыс и « срыв » нервной деятельности производились по методу Горшелевой (119).

После выработки двигательного условного пищевого рефлекса на звонок одновременно со звонком стали включать на 10 сек. электрический ток напряжением в 20-30 вольт. На 8-10-й день такого сочетания двух раздражителей у крыс исчезал условный рефлекс: они по звонку к кормушке не бежали, а забивались в угол и застывали в неподвижной позе. После этого крыс брали для исследования.

Оказалось, что у таких крыс в мозгу снижалось содержание аденозинтрифосфорной кислоты и увеличивалось содержание неорганического фосфора: увеличивалось содержание молочной кислоты, а также интенсивность гликолиза (рис. 11).




Фиг. 11. — Углеводно-фосфорный обмен в ткани головного мозга крыс в норме и при « срыве » нервной деятельности.

Определение относительной удельной активности фосфора АТФ после введения $^{32}P$ показало повышение обновляемости фосфора АТФ.

Нужно думать, что при такой постановке опытов (при срыве) мы имеем дело с перенапряжением нервной деятельности, с чрезмерно сильным истощающим раздражением.

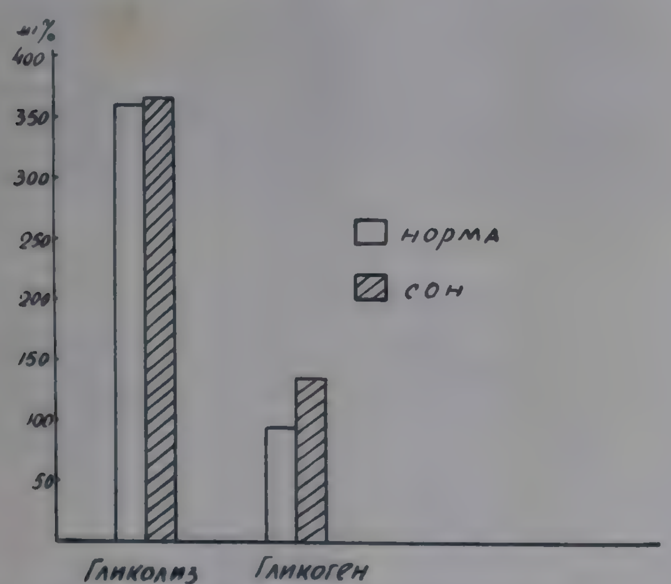
Таким образом, состояние возбуждения нервной системы, вызванное воздействиями, приближающимися к физиологическим, приводят к некоторому (иногда очень незначительному) увеличению содержания АТФ и гликогена наряду с увеличением содержания неорганического фосфора и усилением гликолиза и, судя по увеличению относительной удельной активности, к повышению обмениваемости рибонуклеиновой кислоты, фосфопротеинов и фосфолипидов.

Однако при действии слишком сильных раздражителей и при длительном их воздействии картина изменений в обмене веществ может меняться, так как может наступить истощение нервной системы, или состояние возбуждения может перейти в состояние торможения.

*Обмен веществ при торможении нервной деятельности*

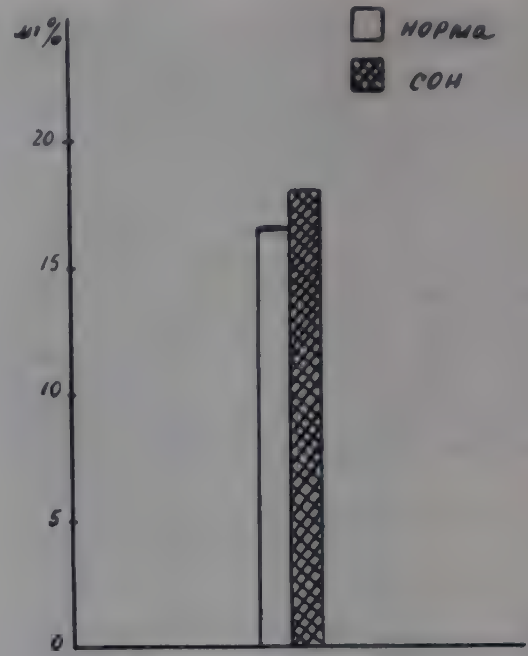
В исследованиях обмена веществ в головном мозгу при торможении нервной деятельности мы применяли мединал натрия или амитал натрия, как фармакологические средства, вызывающие наркотический сон; применяли их в дозировках, приближающих наркотический сон к естественному: находясь в наркотическом сне, животные реагировали на шум, прикосновение и т.п.

Изучение углеводного обмена в мозгу кроликов при 4-часовом наркотическом сне показало, что интенсивность гликолиза была высокой, мало отличаясь от нормы; содержание гликогена было увеличено. Это говорит о том, что обмен углеводов протекает на достаточно высоком уровне, но что их расходование, повидимому, уменьшено (рис. 12). Количество аденоз-




Фиг. 12. — Обмен углеводов в мозгу кроликов при 4-часовом наркотическом сне.

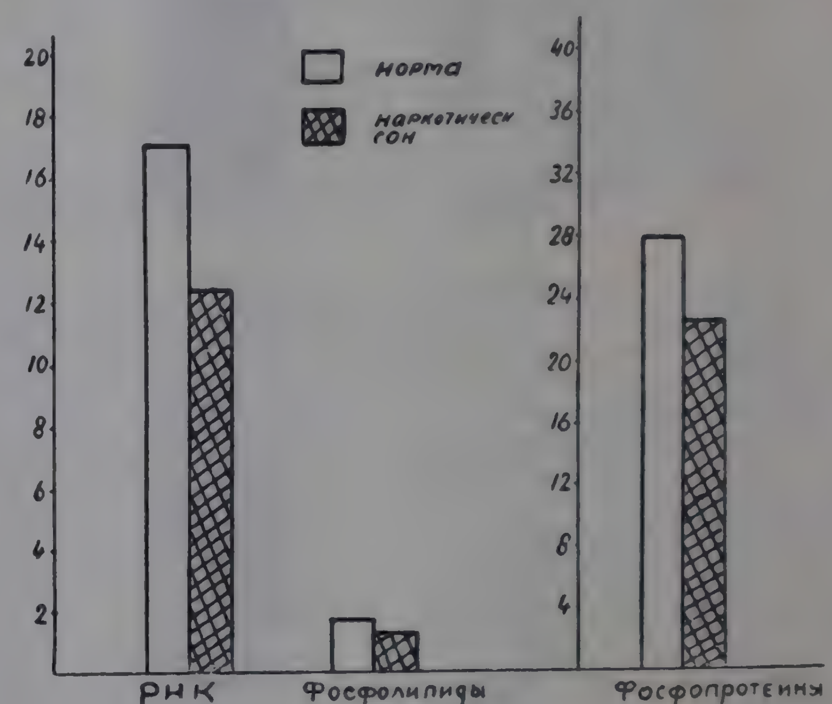
интрифосфорной кислоты при наркотическом сне увеличивается (109) (рис. 13).




Фиг. 13. — Содержание АТФ в мозгу крыс при 4-часовом наркотическом сне.

Что касается нуклеинового обмена, то в содержании нуклеиновых кислот в мозгу крыс при наркотическом сне разной продолжительности резких изменений не наблюдается. В то же время активность фермента дезоксирибонуклеазы возрастает и тем сильнее, чем длительнее сон.

Изучение нами (120) интенсивности включения $^{32}P$ в рибонуклеиновую кислоту, фосфопротеины и фосфолипиды мозга крыс при 24-часовом наркотическом сне (уретан и мединал) показало, что относительная удельная активность фосфора РНК снижалась на 27,6%, фосфопротеинов — на 19,2% и фосфолипидов — на 22,8%; таким образом, при наркотическом сне скорость обновления РНК, фосфопротеинов и фосфолипидов уменьшается (рис. 14).




Фиг. 14. — Влияние 24-час. наркотического сна на относительную удельную активность фосфорных соединений в головном мозгу крыс при введении $^{32}P$ за 2 часа.

Такие же данные получил Владимиров (115), который изучал с помощью радиофосфора влияние наркотического сна (гексанастаб или амитал) на интенсивность обновления фосфора фосфолипидов и рибонуклеиновой кислоты в мозгу крыс и нашел, что при наркотическом сне в головном мозгу животного наблюдается снижение скорости обновления и рибонуклеиновой кислоты и фосфолипидов. При этом в случае амиталового наркоза сон в большинстве случаев был более глубоким и изменения в размерах обновления были более сильно выражены.

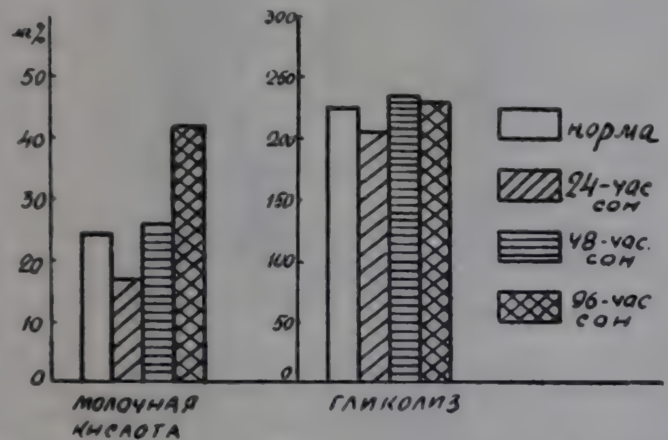
На содержание фосфолипидов в мозгу животного изменение функционального состояния мозга заметного влияния не оказывает.

Таким образом, состояние торможения, вызываемое действием снотворных веществ, характеризуется переходом обмена веществ в мозгу на более низкий уровень: понижается содержание аммиака; понижается интенсивность окислительных процессов, а также интенсивность гликолиза и обновления гликогена; уменьшается обновление фосфора фосфолипидов и рибонуклеиновой кислоты. Содержание аденозинтрифосфорной кислоты повышается, что является подготовкой к предстоящей деятельности после сна.

По Павлову торможение является процессом, охраняющим нервные клетки от истощения и способствующим их восстано-

влению. Наши данные показывают, что при наркотическом сне обмен веществ замедляется, но все же протекает достаточно интенсивно, и что при этом создаются условия для преобладания синтетических процессов, обусловливающих восстановление работоспособности мозга.

Эти изменения наблюдались при непродолжительном сне. Возникает вопрос, останутся ли они такими же при длительном наркотическом сне продолжительностью, например, до 3-х суток (96 часов). Для выяснения этого вопроса Хайкина и Крачко (118) изучили обмен веществ в мозгу крыс при медикаментозном сне, вызванном введением под кожу животного смеси уретана и мединала (два раза в течение суток, различной продолжительностью — 4, 24, 48 и 96 часов). Опыты показали, что при продолжительном сне в мозгу наблюдаются изменения иного порядка, чем при кратковременном сне: содержание молочной кислоты в результате 96-часового сна было не пониженным, а повышенным, содержание АТФ не повышалось, в то время как скорость ее обмениваемости, судя по относительной удельной активности ее фосфора, повышалась (рис. 15). Можно полагать, что эти изменения в обмене веществ мозга при длительном наркотическом сне




Фиг. 15. — Содержание преформированной молочной кислоты, интенсивность гликолиза при наркотическом сне различной продолжительности.

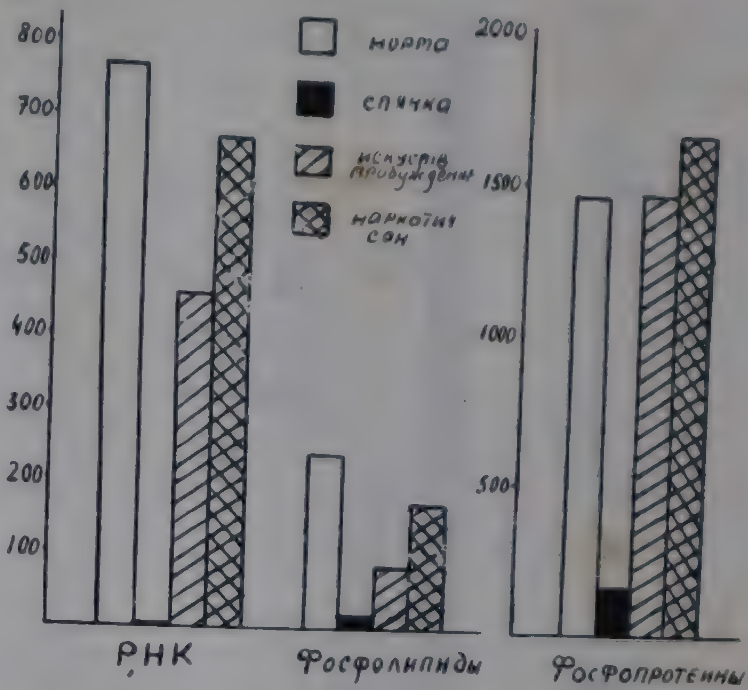
зависят от токсических явлений, связанных с длительным воздействием фармакологических веществ; об этом говорит и болезненный внешний вид животных к концу 96-часового сна.

Считая, что при зимней спячке имеет место состояние длительного угнетения центральной нервной системы, Сквирская и Силич (120) для биохимической расшифровки состояния длительного торможения использовали в качестве объекта исследования зимнеспящих животных, а именно — сусликов. Опыты были поставлены с бодрствующими сусликами, сусликами, находящимися в состоянии спячки, искусственно пробужденными (за 4 часа до опыта), а также с сусликами, находившимися в состоянии 24-часового фармакологического сна.

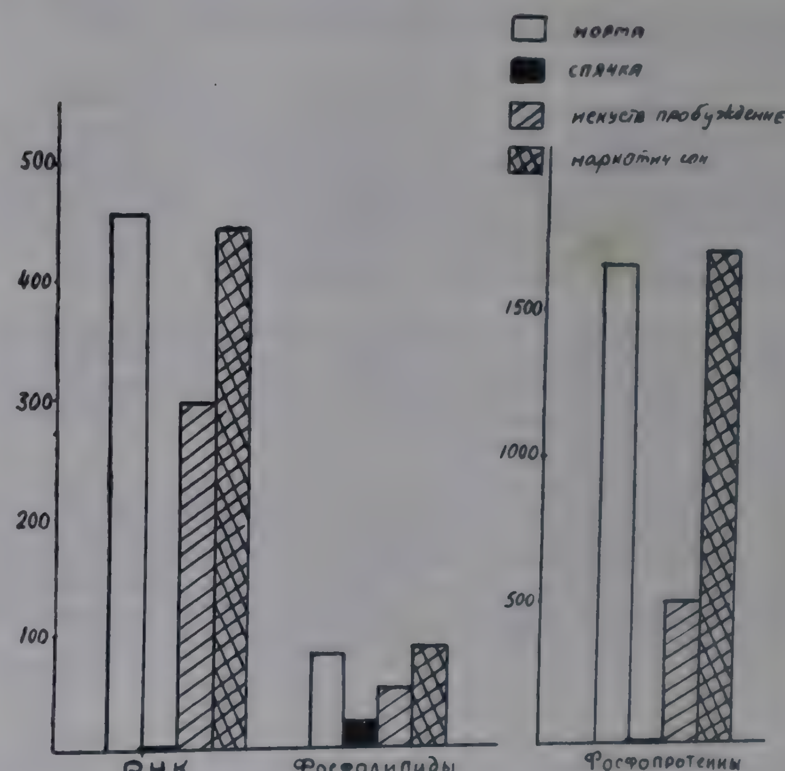
Во всех этих случаях исследовали содержание в головном и спинном мозгу нуклеиновых кислот, фосфопротеинов и фосфолипидов, а также скорость включения в них радиоактивного фосфора, который вводился животным подкожно за 4 часа до умерщвления животного в количестве 0,1 милликюри на 1 кг веса.

Исследования обнаружили значительные различия в интенсивности включения $^{32}P$ в рибонуклеиновую кислоту, фосфопротеины и фосфолипиды головного и спинного мозга у бодрствующих животных и животных, находившихся в состоянии спячки, тогда как изменения в содержании этих веществ были значительно менее выражены. Удельная активность (количество импульсов на 1 мг фосфора данной фракции) для различных фосфорсодержащих соединений в головном и спинном мозгу сусликов при спячке была в несколько десятков раз ниже, чем при бодрствовании, а для некоторых веществ — практически равна нулю (рис. 16 и 17).

При 24-часовом наркотическом сне (мединал натрия) интенсивность включения $^{32}P$ в изучаемые вещества мозга сусликов по своей направленности была подобна той, которая наблюдалась при спячке, но степень снижения интенсивности была на много меньше.



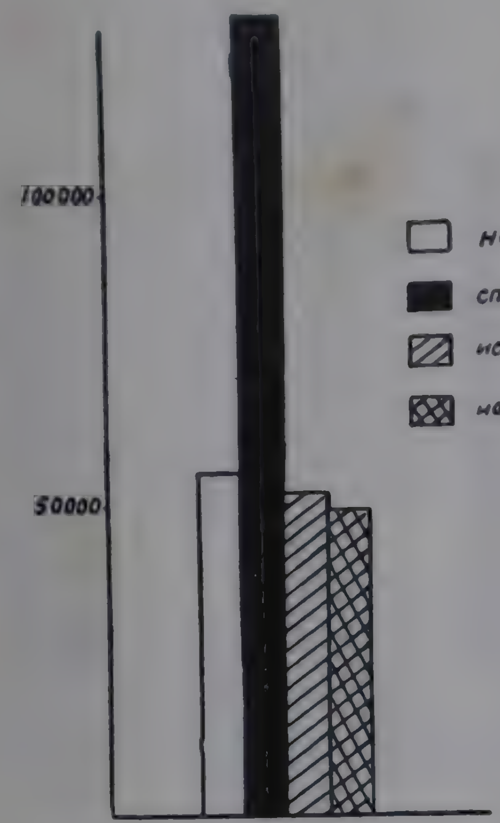

Фиг. 16. — Удельная активность фосфорных соединений в головном мозгу сусликов (имп./1 мг Р) при введении $^{32}P$ за 4 часа.




Фиг. 17. — Удельная активность фосфорных соединений в спинном мозгу сусликов при введении $^{32}P$ за 4 часа (имп. /1 мг Р).

Особенно интересны данные, полученные на искусственно пробужденных животных. При пробуждении интенсивность включения $^{32}P$ в нуклеиновые кислоты и фосфолипиды и другие фосфорные фракции мозга возрастала по сравнению с нормой, однако не достигала уровня бодрствующих животных и животных, подвергнутых наркотическому сну. Пробуждение зимнеспящих животных связано с резкой сменой состояния разлитого торможения функциональной активностью мозга. Весьма вероятно, что при искусственном пробуждении имеет место столкновение этих двух процессоф с развитием запредельного торможения.

Использование меченого фосфора позволило также установить, что проникновение фосфора из крови в ткани, в частности в нервную ткань, чрезвычайно замедлено, с чем, очевидно, в значительной мере связано резкое снижение обмениваемости при зимней спячке (рис. 18 и 19).



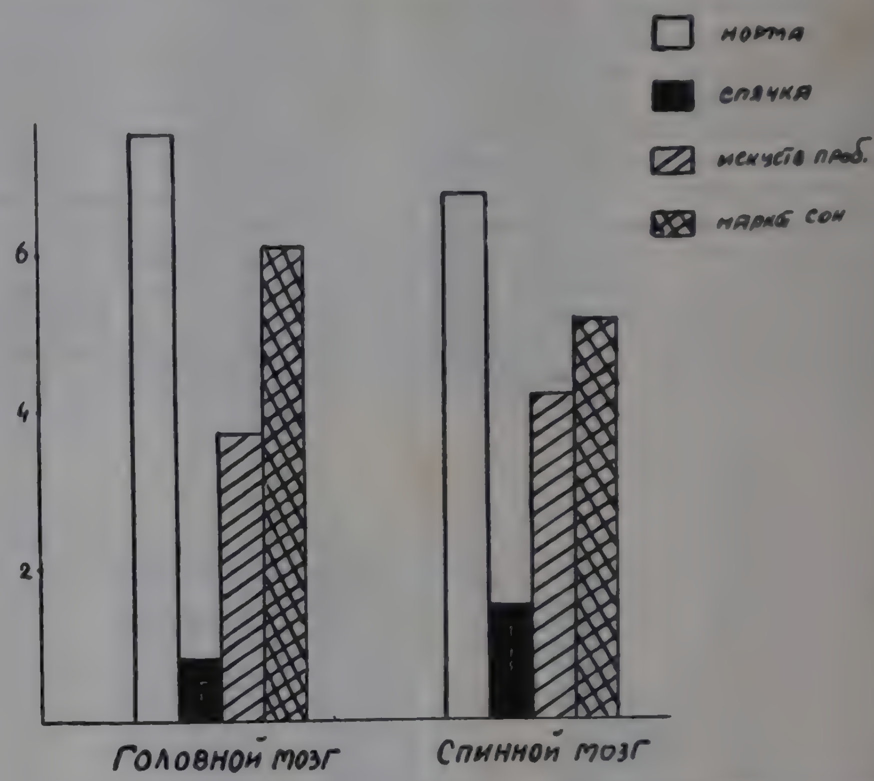

**Фиг. 18.** — Удельная активность кислоторастворимой фракции сыворотки крови сусликов (имп./мг P) при введении $^{32}P$ за 4 часа.

Таким образом, при торможении нервной деятельности (при наркотическом сне, при зимней спячке) процессы распада замедляются, снижается скорость обновления гликогена, рибонуклеиновой кислоты, фосфолипидов, понижается содержание аммиака, увеличивается содержание гликогена и АТФ; создаются лучшие условия для процессов синтеза, что и обеспечивает восстановление работоспособности мозга.

Таковы основные результаты ряда новейших исследований, в том числе и наших, по биохимии головного мозга, в частности по изучению обмена веществ головного мозга в связи с его различным функциональным состоянием и с условиями внешней и внутренней среды.

В своем докладе, стесненный его размером, я не мог охватить всех сторон биохимии головного мозга; многие работы, касающиеся интересных вопросов обмена веществ в мозгу, как, напр., энергетического обмена, окислительного фосфорилирования, остались в моем докладе не освещенными.

Однако и из материала, изложенного в докладе, можно видеть, что сделано уже немало и в деле изучения химического строения функционально различных отделов центральной нервной системы, и в изучении процессов обмена веществ, особенно углеводного обмена, в головном мозгу, и в разработке вопросов функциональной биохимии головного мозга, в частности биохимии возбуждения и торможения нервной деятельности — этих основных физиологических состояний




**Фиг. 19.** — Относительная удельная активность кислоторастворимой фракции в головном и спинном мозгу сусликов: $\left(\frac{\text{уд. акт. к.-р. фр.} \times 100}{\text{уд. акт. к.-р. фр. сыв. крови}}\right)$ при введении $^{32}P$ за 4 часа.

нервной системы. Исследования этого рода показали, что состояния возбуждения и торможения сопровождаются разнообразными и, в общем, противоположными изменениями в обмене веществ.

Несмотря на эти успехи, многое еще остается неизученным. Перед функциональной биохимией головного мозга лежит еще широкое поле исследований, пока окончательно не будут раскрыты биохимические основы специфической деятельности различных частей головного мозга, пока мы не овладеем обменом веществ в головном мозгу настолько, что сумеем управлять им.

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# Measurement of reaction rates in various cerebral systems

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(*Received* 5 *August* 1955)

I have very much appreciated Professor Palladine's account, welcoming the attention which he pays to material changes in the brain in association with mental phenomena, and to the investigation of protein constituents. As Professor Palladine says, the afternoon's discussion can cover only a few of the many aspects from which work is now being contributed to cerebral biochemistry. I have chosen therefore to emphasize one aspect which is complementary to those of Professor Palladine, commenting shortly on some of the points which he raises. The subject I choose is the speed of chemical change in the brain, for it links a great variety of biochemical studies of cerebral processes (1).

The last few years have greatly changed our impressions of the speed of reactions in cerebral systems. In lipids, in phosphates, and in carbohydrate intermediates, reactions have been described which proceed fully 100 times faster than those previously observed in the same category of substance. In this sense, the present period has been a record-breaking one : we will not see another one like it in this subject. However, knowledge of the speed of chemical change in cerebral systems can be very important even when the rates are not extremely high. My observations, necessarily brief, are grouped under four different headings.

*Reaction speed as linking phenomena* in vivo *and* in vitro

I begin with the contribution which measurement of reaction velocity has to make to indicating to the biochemical worker with separated cerebral systems whether or not he is working under conditions relevant to the brain *in vivo*. Recent years have seen many applications of Schmidt and Kety's technique for determining cerebral blood flow and hence metabolic rates. The respiratory rates are stable in a variety of physiological conditions, but fall notably during anaesthesia and in hypoglycaemia. Cerebral respiration also falls in a variety of pathological conditions in which cerebral functioning alters ; but is unchanged in schizophrenia.

Considering now their actual rates, the normal value of 3.3 or 3.5 ml. $O_2$/100 g. tissue/min. found by Kety and Schmidt, corresponds to 90 μ-moles $O_2$/g./h. This implies rates of about 120 μ-moles/g./h. for human cerebral grey matter, and 60 μ-moles/g./h. for white. Actual rates measured for separated human cerebral tissues *in vitro* under ordinary conditions are less than half these values (2, 3, 4) but with several stimulating agents can reach the *in vivo* levels. Most of the stimulating agents (30 mM-KCl, or 2, 4-dinitrophenol) depart from conditions *in vivo*, but one agent (applied electrical pulses) restores

to the tissue an element of its normal environment which ordinary techniques do not supply. Grey matter of the cerebral cortex so stimulated reached 110 μ-moles $O_2$/g./h. and white, 45 μ-moles/g./h. With this approach to *in vivo* rates comes also a greater sensitivity to agents which affect the brain *in vivo* (4, 1).

Glycolytic rates *in vivo* are normally low; lower than those of separated tissues as ordinarily studied with many times their volume of fluid. But by supplying only a little fluid *in vitro*, rates become comparable to those normal *in vivo*. This emphasizes an artificiality in the ordinarily employed tissue-fluid relationships.

Excitation gives extremely high glycolytic rates *in vivo* (5) which only recently have been reproduced *in vitro* through studying the tissue in an electrically excited condition for periods of a few seconds only (6). This emphasizes how intracellular substrates are involved, for the rates of glycolysis appear to exceed those of the entry of glucose to the brain.

### *Reaction speed providing a basis for observing pathologically or experimentally-induced abnormality*

I will make only very brief mention of this theme. Observations by arterial-venous difference have already been noted. Also important are the abnormal respiratory or glycolytic rates reported in cerebral neoplasms and in separated cerebral tissues in mental disease (7, 8) and abnormal rates of acetylcholine metabolism in tissue from epileptogenic areas (9).

### *Reaction speed in understanding co-ordinated metabolic processes*

An outstanding example concerns the major oxidative route in cerebral tissues. Since 1940, evidence that this is indeed the tricarboxylic cycle has grown, the most recent coming from the elegant elucidation especially in Peters' laboratory, of the action of fluorocitrate (10, 11). It is necessary, however, to know whether this process is quantitatively adequate. A recent assessing of published data in individual enzyme rates shows that this is indeed the case (1). Moreover, although many of the rates concerned are the result of recent investigations and others may still be subject to revision, some pattern can be seen in their actual magnitudes. Observed rates for stages of the cycle itself are all higher, some enormously higher, than those of the processes bringing pyruvate into the cycle. The entry occurs at some 30 to 50 μ-moles pyruvate/g./h., corresponding to respiratory rates of 75 to 125 which are just those observed. But rates of the later stages are up to 2000 and 5000 μ-moles/g./h. (12, 13). Such relationships are probably major factors in keeping the cycle functioning as a cycle; I would judge them to be of equal importance in this respect to the structural association of the enzymes concerned at the mitochondria.

Unfortunately these enzymic levels have not yet been expressed in terms of the quantity of enzyme in the cell; but knowledge that rates over 5000 μ-moles/g./h. do occur in the tissue is an important extension of our information about it; knowledge giving perspective to other processes; for example, the maximum rate observed in acetylcholine synthesis in cerebral tissues is about 1 μ-mole/g./h. Several phosphokinases also, however, proceed at some thousands of μ-moles/g./h. (14, 15, 16).

### *Reaction speed in helping to assess the significance of isolated metabolic processes*

Our knowledge of cerebral biochemistry includes not only processes observed *in vivo* but of unknown mechanism; it also includes what may be suspected to be parts of the enzymic mechanism of processes important *in vivo* but as yet unidentified. To the assessment of such reactions, knowledge of their velocity and specificity greatly contribute. Why does the cerebral diphosphoinositide undergo such rapid degradation ? At 300 μ-moles/g./h. this reaction is several times the respiratory rate of the tissue, and 100 times that of rates observed in other lipids by cerebral tissues (17, 18). The formation of ammonia is another such process : it can occur at 450 μ-moles/g./h. (19) which is markedly greater than the rates of known reactions leading to its formation.

### *Envoy*

I conclude with brief reference to Professor Palladine's opening, agreeing it important to study biochemically the intact higher organism; but emphasizing this is not more important or productive than many other chemical and biochemical approaches to them. Study of separated tissue has more than auxiliary value; in it, and in subcellular particles, are systems of much complexity and autonomy : characterizing, limiting, conditioning, the organs into which for expediency they now form themselves. Biochemical mechanisms can be regarded as of greater significance than the organisms in which they are at present manifested, which commonly allow only partial and occasional display of tissue potentialities; display becoming however greater and more continuous as in our higher organisms the whole caters for its parts by salt, glucose, temperature and other regulatory mechanisms. These are complex processes with which the brain concerns itself, weaving cells, fibres, and secretory mechanisms to cater for the general chemical properties of bodily proteins and for the evolutionary route which chose mitochondria; elaborating probably not incidentally the only known nidi for many interesting compounds to display their potentialities. For purposes of investigation it is important to remember that metabolism and function are mental isolates; that the successful organism has great respect for the individual compounds and tissues which it serves and whose study, therefore, is an important way of learning what the organism is about.

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# The metabolic activity of the proteins of the brain

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(*Received* 5 *August* 1955)

The broad physiological viewpoint taken by Prof. Palladine in his report on the biochemistry of the brain will be welcomed by many who are working in this field. While acknowledging the value of metabolic studies *in vitro*, he has emphasized the importance of carrying out parallel studies *in vivo*. Such work can help to show the relation of metabolism to the physiological events occurring in the living organism as a whole.

Another welcome feature of Prof. Palladine's report is the amount of interesting new Russian work that he has been able to discuss. Much of the Russian work in this field is still relatively little known to those working in other countries.

### *The metabolic activity of the proteins of the brain*

Prof. Palladine has referred to some work carried out at Cardiff in which evidence was obtained of the metabolic activity of the proteins of the brain by measuring the rate of incorporation of a labelled amino acid *in vivo* (1). This work has now been extended by Dr. Gaitonde, who has obtained a number of new results that appear to be relevant (2). The amino acid used in these experiments was the naturally-occurring L-isomer of $^{35}$S-methionine. When $^{35}$S-methionine was given by intraperitoneal injection it was found, in agreement with previous work by other investigators, that the rate of uptake into the brain was small. If, however, the labelled amino acid was introduced directly into the cerebrospinal fluid (CSF) by intracisternal or subarachnoid injection, the rate of uptake in the brain was relatively high. This therefore offered a means of investigating the metabolic activity of the brain proteins under conditions more favourable for observing the actual rate of amino acid incorporation.

Experiments carried out to determine how the $^{35}$S was distributed in the different fractions of the brain tissue showed that at 3 hours after injection over 80 % was in the proteins. Of this about 76 % was present as $^{35}$S-methionine and 24 % as $^{35}$S-cysteine. The $^{35}$S-cysteine was apparently combined in normal peptide bonds. Both were isolated from the brain proteins and shown to have the same radioactivity on recrystallization.

From the rate at which the proteins acquired $^{35}$S by the incorporation of $^{35}$S-methionine, an estimate of the normal rate of replacement of methionine in the rat brain *in vivo* could be made. It was assumed that the relatively large amount of $^{35}$S-methionine (1 mg.) introduced into the CSF by injection was sufficient to swamp the small amount of methionine normally present in the amino acid 'pool', so that the specific activity of the methionine incorporated was similar to that of the methionine injected. Measurements of the minimal rate of incorporation of methionine into the brain proteins in the first 20-60 minutes after intracisternal or subarachnoid injection gave a mean rate of 0.41 microequivalents per gram protein per hour. This is considerably higher than the values obtained by previous investigators who did not take the blood brain barrier into account (3). Expressed in other terms, the rate of methionine incorporation *in vivo* indicated a half-life of the order of 14 days for the methionine bonds in the brain proteins. This may be compared with Sprinson and Rittenberg's (4) estimate of 6 days for the half-life of the proteins of the visceral organs and 21 days for the carcass proteins in the rat. Since the tissue proteins are not homogeneous, calculations of half-life are of limited significance, but taking the half-life of the methionine bonds in the brain proteins as 14 days, that would imply a half-life of less than one day for a whole protein molecule of 80 000 molecular weight containing 15 methionine units.

It would appear that the different amino acids do not all behave in the same way in relation to protein formation and some evidence of this is being reported in a paper given by Clouet (5). But if similar considerations are applied to the bonds of the 20 other amino acids and to the labile bonds of the phospholipid units (6), it would

appear that the 'half-life' of some at least of the lipoproteins, which are the main organic constituents of the brain, must be of the order of seconds rather than days.

*Factors affecting amino acid incorporation in the brain proteins*

Prof. Palladine has mentioned some very interesting unpublished experiments from his laboratory on the effect of vitamin deficiencies on amino acid incorporation. We have experienced some difficulty in making comparisons between different animals in different physiological states owing to the large individual variation and the difficulty of administering a strictly uniform dose of $^{35}$S-methionine by the intracisternal route. These difficulties can be to a large extent avoided if, instead of trying to measure the absolute rate of uptake of $^{35}$S in the proteins, we relate that to the amount entering the acid soluble phase of the tissue and measure the specific activity ratio. That, in effect, corrects the value for variation in the amount of $^{35}$S-methionine entering the tissue. Using the ratio as an index of the uptake of $^{35}$S into the tissue proteins, it was found that this shows a significant relation to age. The ratio was considerably higher in young animals than in the adult.

The comparison by similar methods of groups of animals in different physiological states showed a significantly decreased rate of amino acid incorporation in ether and in pentobarbitone narcosis. The effect was more pronounced if the temperature of the animals was allowed to fall. Insulin hypoglycaemia also produced a significant fall in the amino acid incorporation. Contrary to expectations, electrical stimulation of the brain produced very little change. If anything, there was again a slight fall in the rate of amino acid incorporation.

Another problem of considerable interest is the distribution of protein formation in different parts of the brain. Dr. Gaitonde is giving a paper on this in which he is going into this matter in some detail (7), and it may be sufficient to mention here that direct chemical estimations agree with results obtained by the autoradiographic method in indicating a high degree of specificity in the rate of amino acid incorporation in different parts of the brain.

It is known that brain tissue contains a number of active peptidases and other enzymes concerned in protein metabolism (8). Taken together, there is thus considerable evidence that the proteins of the brain *in vivo* are metabolically active, and that the activity varies with the physiological state.

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# Cellular structure and enzymic bacteriolysis

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(*Received* 8 *April* 1955)

Cytologists are now in general agreement as to the main structural features of the bacterial cell. Most bacteria possess a rigid cell-wall structure of different properties to the enclosed cytoplasm and in this respect they closely resemble cells of higher plants. Many micro-organisms have capsular substances or slime layers outside the cell wall. By analogy with the structure of higher organisms, it has been held that the bacterial cell possesses a well-defined membrane which may be differentiated from its external neighbour, the cell wall and from the inner cytoplasm. However, the proof of the existence of a cytoplasmic membrane has been less satisfactory than for other bacterial structures. More convincing and direct evidence for the presence of a membrane in at least some bacteria has been provided by Weibull's experiments (1).

Our knowledge of the structure of the bacterial cell has been gained from the studies of the interaction of cells with dyes, surface-active agents, antibodies, enzymes etc. Substances such as the dyes and surface-active detergents react with cellular components of great molecular diversity and can only give us a limited amount of information about specific structures. Owing to their more circumscribed spheres of action, enzymes and antibodies have provided valuable systems for determining the nature and location of specific cellular components. Thus a knowledge of the chemical constitution of the substrates attacked by certain enzymes has furnished us with an elegant means of cytochemical analysis of the bacterial cell.

In considering enzymes as analytical tools in the cytochemical study of bacterial structure it seems pertinent that the enzymes should be grouped according to the location and nature of the structures they degrade. On this basis they may be conveniently classified into four groups :

(1) Enzymes removing superficial capsular or slime layers from bacteria without affecting the viability of the organisms.

(2) Lytic enzymes having a direct action on a structure or structures responsible for the morphological integrity of the cell, *e. g.* enzymes digesting cell walls.

(3) Proteolytic enzymes bringing about the lysis of heat-killed bacteria.

(4) Enzymes responsible for secondary lysis of cells as a result of the killing action of physical, chemical or antibiotic agents ; this group probably includes proteolytic enzymes as well as the ill-defined collection known as the « autolytic enzymes ».

The enzymes involved in group 4 are far from being fully characterized and bacteriolysis probably represents the culminating effects of the activities of a multiplicity of enzymes. It seems likely that some autolytic enzymes may be shown to have a direct action on cell-wall structures. Enzymes in group 1-3 have yielded the most valuable information about specific cellular components and of these enzymes, the bacteriolytic systems have been widely studied. Bacteriolytic enzymes from the tissues and secretions of a great variety of organisms have been investigated and those of micro-organisms have received much attention and the extensive literature on the lytic factors of microbial origin has been admirably reviewed by Waksman (2) and Welsch (3).

### *Enzymic digestion of capsular and surface components of the bacterial cell*

It is now well known that many bacteria possess capsules or superficial layers of substances not intimately associated with the structural integrity and viability of the cell. Dubos and Avery (4) isolated a spore-forming bacillus on a medium containing type III pneumococcus capsular polysaccharide and obtained enzyme preparations capable of degrading the polysaccharide. When intact, living type III pneumococci were treated with the enzyme, the capsular polysaccharide was removed without impairing the viability of the cells (5). In a similar fashion Lancefield (6) demonstrated that the removal of the M-protein antigen from haemolytic streptococci by treatment with trypsin was achieved without death of the organisms. Treatment of streptococci with hyaluronidase increases their susceptibility to phagocytosis (7) and removes a component (presumably hyaluronic acid) blocking bacteriophage attachment and infection (8). Enzymes capable of hydrolysing the isolated γ-glutamyl peptide capsule of *Bacillus subtilis* have been reported (9, 10) and it will be of interest to see if the capsules can be removed from intact bacteria by exposure to these enzymes.

Although such enzyme studies as these do not fall into the strictly bacteriolytic class, I believe they are sufficiently important to warrant attention here. A completely negative response to treatment of intact, encapsulated bacteria with a lytic enzyme is insufficient evidence to conclude that there are no underlying structures susceptible to digestion with bacteriolytic enzymes. Thus enzymic hydrolysis of capsular substances of some organisms may of necessity preceed a study of the action of truly bacteriolytic systems.

### *The action of bacteriolytic enzymes on cell walls*

#### *Lysozyme*

In 1922 Fleming (11) observed the lysis of *Micrococcus lysodeikticus* by a powerful bacteriolytic agent found in tissues and secretions. This substance was called lysozyme and since its discovery its lytic properties have been widely investigated. The relative ease with which egg-white lysozyme may be crystallized (12) has made it an attractive lytic enzyme to study and has greatly facilitated our present understanding of its action on the bacterial cell.

Meyer *et al.* (13) were the first to study the mechanism of the action of lysozyme and their investigations showed that lysozyme liberates reducing substances from bacterial mucoids. Meyer (14) concluded that lysozyme action could be « explained in terms of hydrolysis of a substance of mucoid nature which is contained in the bacterial membrane ». Epstein and Chain (15) suggested that the polysaccharide attacked by lysozyme was an essential element for the maintenance of the morphological structure of sensitive bacteria and that it was present in the intact organisms in an insoluble form. Boasson (16) on the other hand concluded that the cell wall of *M. lysodeikticus* was not destroyed by the action of lysozyme but that it became more permeable to the cellular contents.

Before discussing more recent developments in the study of the mechanism of lysozyme action, two properties of the substance lysozyme must be kept in mind when we consider its interaction with bacterial cells. Firstly, it is an enzyme capable of hydrolysing certain mucocomplexes; lysis and liberation of reducing substances are the two manifestations most commonly observed. Secondly, it is a strongly basic protein and as such it is capable of forming complexes with negatively charged substances *e. g.* anionic surface-active compounds (17). Properties that are associated with the physico-chemical nature of lysozyme in contradistinction to its enzymic capabilities include its ability to agglutinate bacteria (18) and increase the turbidity of certain heated bacteria in a similar fashion to the basic protein, salmine. The observation that at high concentrations of lysozyme there is a substantial reduction in lysis (20) may represent the summation of its enzymic and physico-chemical properties. There seems to be an unfortunate tendency to overlook the possibilities that some of the effects observed with lysozyme could be duplicated with non-enzymic basic substances and this has led to much confusion as to the susceptibility of various bacteria to the enzymic action of lysozyme.

With the development of suitable methods for the isolation of bacterial cell walls (21) a more direct approach to the study of the mechanism of lysozyme action was made possible. When the isolated cell walls of *M. lysodeikticus* were incubated with crystalline egg-white lysozyme, complete dissolution of the walls occurred and no residual wall-like structures could be detected on examination in the electron microscope (22). In a similar way the complete degradation of the isolated walls of *Bacillus megaterium* and *Sarcina lutea* by lysozyme has also been established (19, 23). Using different techniques, several workers in other laboratories have demonstrated the action of lysozyme on the rigid cell-wall structures. By combined phase-contrast microscopy and immunological reactions, Tomcsik and Guex-Holzer (24) concluded that lysozyme acted specifically on the cell wall of a *Bacillus* sp. Welshimer (25) using crushed bacterial preparations observed the dissolution of wall fragments on treatment with lysozyme.

It seems that one of the most sensitive and certain methods of determining whether bacterial structures are degraded by lysozyme is to follow turbidimetrically the interaction of the isolated walls with lysozyme. As illustrated in figures 1 and 2, the rates of dissolution of the walls by crystalline egg-white lysozyme differ from one bacterium to another. Thus *M. lysodeikticus* walls

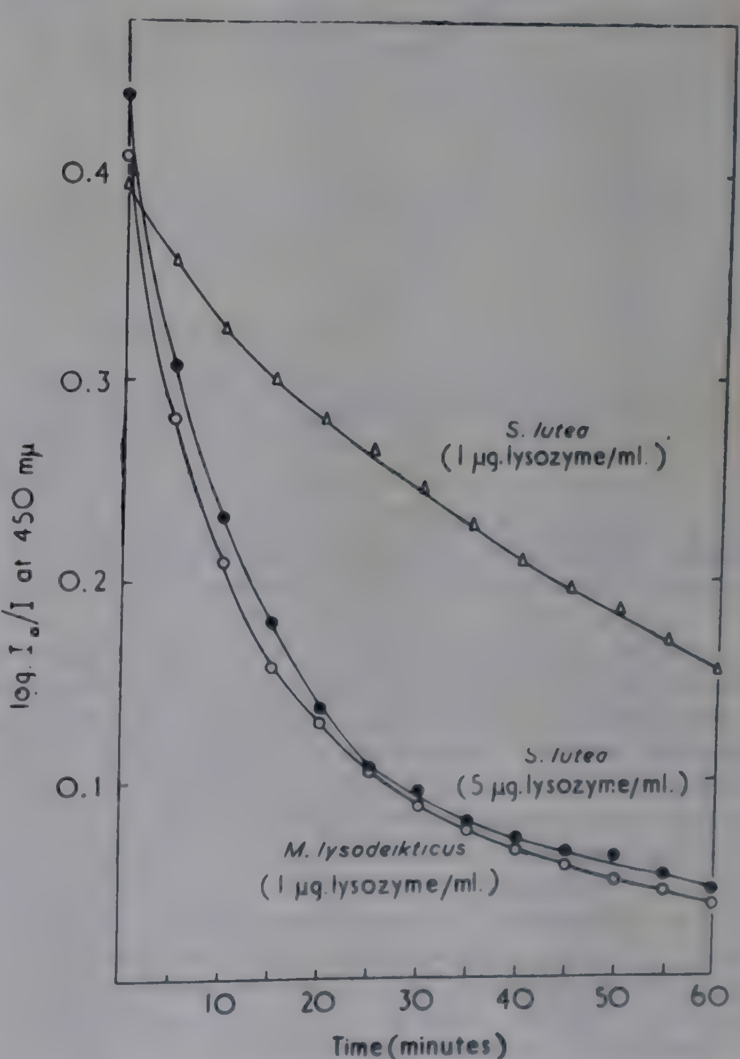


FIG. 1. — Lysis of bacterial cell walls by crystalline egg-white lysozyme. Incubation at 37° C. in 0.1 M phosphate buffer pH 6.0. Optical densities of approximately 0.4 (log $I_0/I$ at 450 mμ) correspond to 1.0-1.5 mg. dry weight bacterial cell wall/ml.

are rapidly lysed on incubation with 1 µg. lysozyme/ml.; with the same amount of lysozyme, *S. lutea* cell walls are dissolved more slowly and as shown in figure 1 higher concentrations of lysozyme are required for more rapid lysis. Such differences may be due to the frequency and location of the linkages attacked by lysozyme, differing in the walls of the two organisms. For the dissolution of the cell walls of *Bacillus* spp. much higher concentrations are required. The lysis curves in figure 2 show

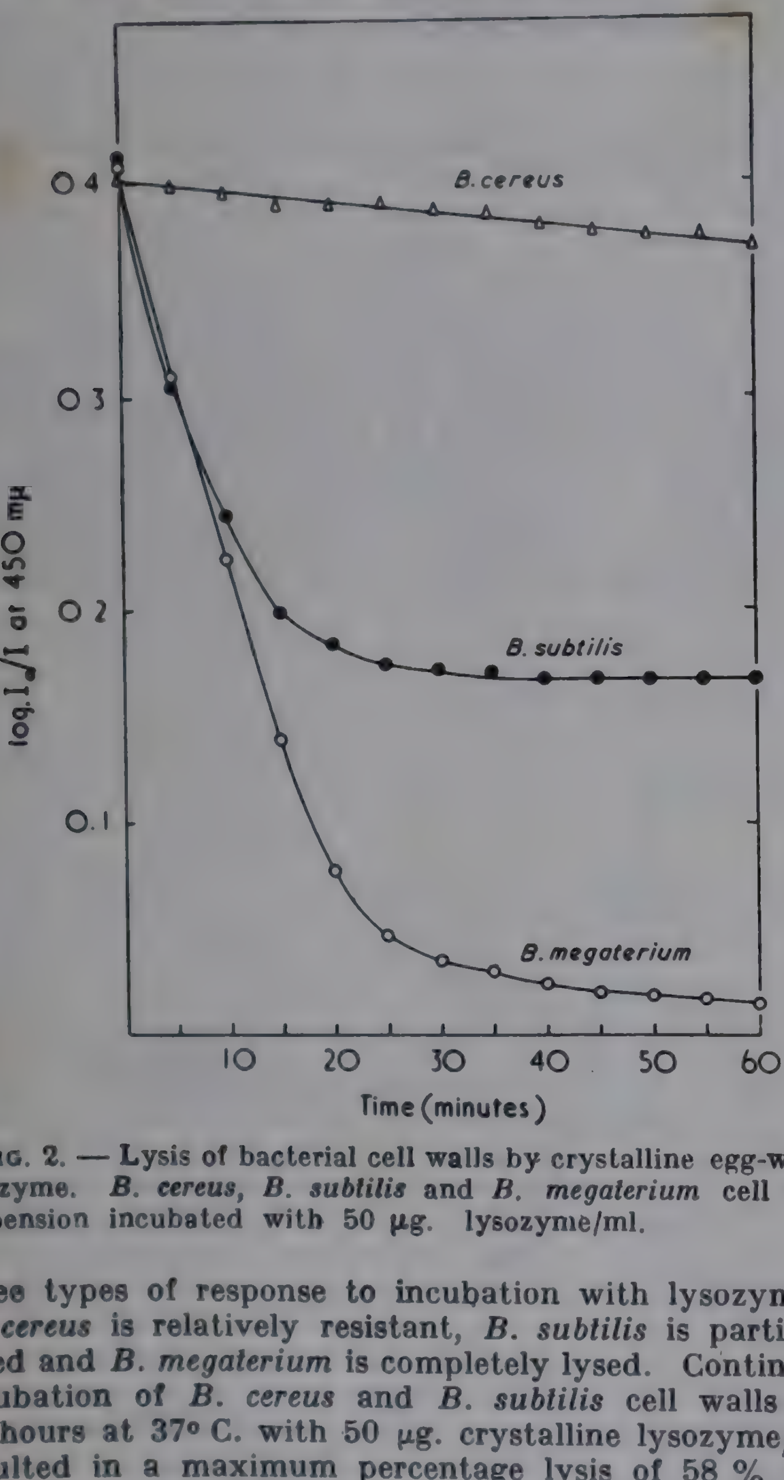


FIG. 2. — Lysis of bacterial cell walls by crystalline egg-white lysozyme. *B. cereus*, *B. subtilis* and *B. megaterium* cell wall suspension incubated with 50 µg. lysozyme/ml.

three types of response to incubation with lysozyme: *B. cereus* is relatively resistant, *B. subtilis* is partially lysed and *B. megaterium* is completely lysed. Continued incubation of *B. cereus* and *B. subtilis* cell walls for 10 hours at 37° C. with 50 µg. crystalline lysozyme/ml. resulted in a maximum percentage lysis of 58 % and 57 % respectively. Examination of the residues in the electron microscope revealed the presence of residual wall-like structures. These observations support the suggestion (15) that certain bacteria possess other structural components in addition to those dissolved by lysozyme. On the basis of the chemical analysis of the cell wall of *B. subtilis* this would have been anticipated (26).

Earlier investigations with the soluble substrate isolated from lysozyme-sensitive bacteria (13, 15, 27) established its mucopolysaccharide nature. The chemical constitution of the isolated walls of several lysozyme-sensitive bacteria have been studied (23, 26) and they fall within the general classification of mucocomplexes, *i. e.* they contain polysaccharide components as indicated by the liberation of reducing substances on acid hydrolysis and amino-acid containing moieties. Owing to the rather restricted variety of amino-acids in these cell walls (26) the term « protein » should be avoided in the description of the amino-acid containing components. Some of the properties of the cell walls attacked by lysozyme together with several lysozyme-resistant components of microbial origin are listed in table I.

The most outstanding characteristic revealed in table I is the presence of the two reducing substances, glucose and hexosamine, in all four bacterial cell walls susceptible to digestion with lysozyme. The exact nature of the hexosamine has not been fully established, but recent evidence (28) obtained with Gardell's method (29) for the identification of amino-sugars, indicated that most of the hexosamine colour reaction of *M. lysodeikticus* cell-wall hydrolysates can be accounted for by glucosamine and that no galactosamine is present. Apart from the reducing substances, the similarities of the amino-acid constituents shown in table I are less striking although there may be some significance in the small variety of amino-acids in the most sensitive organisms (*M. lysodeikticus* and *S. lutea*) and the greater variety in the walls of the two *Bacillus* spp. The lysozyme resistant *Streptococcus faecalis* cell wall containing rhamnose, glucose, galactose and hexosamine (30) and the glucose-galactose capsular polysaccharide isolated from a strain of *B. megaterium* by Aubert (31) (Dr. Aubert kindly provided a sample of the polysaccharide) differ chemically from the substrates attacked by lysozyme. It seems then that a reasonable tentative conclusion would be that the specificity of lysozyme is associated with the glucose-glucosamine components.

As may have been expected from the mucopolysaccharide nature of the walls of these organisms, the products of lysozyme digestion are complex. The complexity of the end products of lysozyme action has been revealed by examination of digests in the ultracentrifuge and by electrophoretic studies. A preliminary investigation of *M. lysodeikticus*, *S. lutea* and *B. megaterium* lysozyme-digested walls in the ultra-centrifuge has shown that the major components of the wall digests are polydispersed and have molecular weights of the order of 10 000-20 000 (23). Electrophoretic examination of the non-dialysable fraction from lysozyme digested *M. lysodeikticus* wall showed three main components. One of the components did not migrate when electrophoresis was carried out over the range pH 2.0-8.5. Such a neutral component may be an oligosaccharide.

Dissolution of the walls with lysozyme is accompanied by a liberation of reducing substances and material giving a positive reaction for acetylhexosamine. The earlier suggestions that lysozyme liberates free N-acetylhexosamine (15, 27) have not been substantiated. Although the absorption spectrum of the colour complex given by the cell wall digests and the acetylhexosamine reagents is identical to that of N-acetylglucosamine, no free N-acetylglucosamine could be detected by paper chromatography (23). No free glucose (the other reducing substance in the walls) nor any free amino-acids could

TABLE I

*Properties of bacterial cell walls and a capsular polysaccharide and their reactions to treatment with crystalline egg-white lysozyme*

| | Constituents identified in hydrolysates | | Reaction to lysozyme treatment (*) |
|---|---|---|---|
| | Reducing substances | Amino-acids | |
| Cell walls | | | |
| *M. lysodeikticus* | glucose, hexosamine | alanine, glutamic acid, glycine, lysine | lysis and liberation of reducing substances (10.7 %) (**) |
| *S. lutea* | glucose, hexosamine | alanine, aspartic and glutamic acids, glycine, lysine | lysis and liberation of reducing substances (3.4 %) (**) |
| *B. megaterium* | glucose, hexosamine | alanine, aspartic, glutamic and diaminopimelic acids, serine, glycine, valine, lysine | lysis and liberation of reducing substances (6.7 %) (**) |
| *B. subtilis* | glucose, hexosamine | alanine, aspartic, glutamic and diaminopimelic acids, serine, glycine, threonine, lysine, valine, leucine | partial lysis and liberation of reducing substances (4.2 %) (*) |
| *Strep. faecalis* | glucose, galactose, rhamnose, hexosamine | alanine, aspartic and glutamic acids, serine, lysine, glycine, threonine, valine, leucine | No lysis and no detectable reducing substances |
| Capsular polysaccharide (31) | | | |
| *B. megaterium* | glucose, galactose | — | no liberation of reducing substances |

(*) 50 µg. lysozyme/ml., 0.1 M $NH_4Ac.$, pH 6.3; 24 h. incubation at 37° C. microbial substrates 15 mg. dry wt./ml.
(**) Reducing substances liberated (glucose equivalent expressed as % dry weight cell wall).
Data shown in this table taken from 23, 26, 28, 30, 31.

be detected in the digests. However, chromatography of concentrated dialysable material from lysozyme digests of *M. lysodeikticus*, *S. lutea* and *B. megaterium* revealed the presence of a component giving a yellow colour on spraying with acetylhexosamine reagents and a brown colour when the chromatograms were sprayed with aniline hydrogen phthalate. On elution and hydrolysis of the material corresponding to the yellow spot on the chromatograms, a strong hexosamine reaction was obtained. It would seem that this hexosamine-containing substance is the main small fragment liberated by the action of lysozyme.

Although much remains to be done in characterizing the complex collection of products formed by the action of lysozyme on certain cell walls we at least now have some idea of the constitution of several wall substrates and a few indications of the types of end products to be expected.

Apart from egg-white lysozyme, little is known about the activities of other lysozymes or lysozyme-like enzymes. There are of course many reports of micro-organisms, in particular actinomycetes, producing lytic substances active against a wide range of bacteria including lysozyme-sensitive organisms (2, 3). Filtrates of cultures of a number of *Bacillus* species have also contained substances lytic for various organisms (32). More recently Smoliar (33) has studied the bacteriolytic activities of various strains of *B. megaterium* upon one another and has established the presence of a cell-free autolytic system analagous to that found for staphylococci (34). Other evidence for the existence of lytic enzymes similar to lysozyme is the isolation of an antibiotic substance from an actinomycete having similar properties to egg-white lysozyme (35).

With more specific methods available for the testing of the lytic activities of micro-organisms (36), it is now reasonable to conclude that some actinomycetes produce lysozyme-like enzymes; their lytic spectra are very similar indeed to that of egg-white lysozyme. Some recent investigations by Richmond (personal communication) have shown that a *Bacillus* sp. isolated on *M. lysodeikticus* cell walls, produces an enzyme capable of lysing cell wall preparations of *B. megaterium*, *S. lutea* and *M. lysodeikticus*. The observation that the lytic substance produced by this *Bacillus* sp. only partially lyses cell wall preparations of *B. subtilis* shows an even more striking similarity between Richmond's lytic factor and egg-white lysozyme than has been recorded for other microbial lysozyme-type enzymes. It will be of great interest to see whether these lysozyme-type enzymes possess any of the physico-chemical properties of egg-white lysozyme and whether the products of digestion of the cell walls are similar.

*Streptolytic enzyme*

Another bacteriolytic enzyme system that has received attention recently has been that produced by *Streptomyces albus*. Maxted (37) demonstrated lysis of suspensions of β-haemolytic streptococci incubated with the streptomyces enzyme preparation and liberation of the *C* substance. From the concomitant lysis and release of the group specific *C* substance it seemed likely that the streptomyces enzymes were in fact cell-wall hydrolysing enzymes. Experimental verification of the direct action of the streptomyces enzymes on the cell walls of group *A* haemolytic streptococci was provided independently by McCarty (38, 39) and Salton (26, 40).

McCarty (38) has studied in some detail the production and fractionation of the « streptolytic » enzyme system and has investigated the action of the enzyme on bacterial cell wall preparations (39). He has made the important observation that the purified enzyme fractions active against the group *A* streptococci were not lytic for *M. lysodeikticus*. This of course implied that the streptolytic enzyme was quite different from the lysozyme-like enzymes produced by various actinomycetes. A comparison of the chemical constitution of the cell walls of group *A* streptococci (26, 39) with those of lysozyme-sensitive bacteria (23, 26) (see also table I) would support the idea that the streptolytic enzyme is indeed different. Group *A* streptococcal wall contains rhamnose and hexosamine (26, 39) whereas the cell walls of certain lysozyme-sensitive bacteria contain glucose and hexosamine. The only other streptococcal wall studied chemically is that of *Strep. faecalis*; on hydrolysis of the walls, the reducing substances have been identified as rhamnose, glucose, galactose and hexosamine (30). The Streptomyces species isolated on *Strep. faecalis* cell-wall agar also possessed lytic activities on *Strep. agalactiae* cell walls (36). The evidence strongly suggests that the action of purified Streptomyces enzymes may be confined to those walls containing rhamnose and hexosamine as the common reducing substances.

From the investigation of the action of the streptolytic enzyme on group *A* streptococcal cell walls, McCarty (39) concluded that the non-dialysable products were heterogeneous. A portion of the cell wall glucosamine was dialysable after enzymic digestion. The dialysable glucosamine was not free glucosamine and not made up entirely of acetylglucosamine (39). Thus the properties of enzymically digested group *A* streptococcal walls are rather reminiscent of those of the lysozyme digested walls.

The streptolytic enzymes produced by *Streptomyces albus* constitute a second group of cell-wall degrading enzymes. The evidence suggests that the general pattern of enzymic action is rather similar to that of egg-white lysozyme but that the streptolytic enzymes have different specificity requirements. Micro-organisms other than Streptomyces species producing lytic enzymes for streptococcal walls, include *Micromonospora* species and *Nocardia gardneri* (36). In addition to the actinomycetes, a gram-positive, yellow-pigmented coccus has been found to possess lytic activities for *Strep. faecalis* and *Strep. agalactiae* but little or no activity has been observed on *M. lysodeikticus* and *B. megaterium* cell wall preparations (28).

*Other lytic enzymes active against living bacteria*

Welsch (2) has studied the bacteriolytic systems of a number of actinomycetes and has reported the lysis of living *Staphylococcus aureus*. Autolytic enzymes also active against living *Staph. aureus* have also been obtained (41). As these enzymes effect lysis of living cells of *Staph. aureus* it seems probable that they too may be cell-wall degrading enzymes. The chemical composition of the glycerophospho-protein complex of the cell wall of *Staph. aureus* (42) differs markedly from the muco-complexes of the streptococcal and lysozyme-sensitive walls. This difference in composition may suggest that the « staphylolytic » enzymes are distinct from the streptolytic and lysozyme systems. The lytic substance has been purified by adsorption on and elution from the microbial substrates (43) and it will be of great interest to know whether the purified lytic factor has a direct action on the cell walls of *Staph. aureus*.

Greenberg and Halvorson (44) have recently studied an autolytic substance produced during sporulation of *Bacillus terminalis*. The lytic substance rapidly lysed suspensions of young vegetative cells of *B. terminalis* and *B. cereus*; it is of interest to note that *B. megaterium* was not lysed by this substance. It appears likely that this « autolytic » substance may also fall within the group 2 classification (see above) of enzymes having a direct action on cell-wall structures of certain related *Bacillus* species. The disappearance of the sporangium (vegetative cell wall) and liberation of free spores is well known and it is only reasonable to expect that such a process may be achieved by enzymic breakdown of the wall surrounding the mature spore. Some recent observation with a spore-forming strain of *B. megaterium* have shown that the outer vegetative wall does not persist for long after maturation of the spore (28). Thus the rapid disappearance of the sporangium during sporulation would imply enzymic digestion of the vegetative walls.

*Proteolytic enzymes and bacteriolysis*

It has been known for some time that living bacteria are generally resistant to proteolytic enzymes and that dead bacteria are often lysed by such enzymes. When bacteria were killed by heating at 80° C., Kantorowicz (45) noted that the gram-negative organisms were more susceptible to digestion with trypsin than were gram-positive bacteria. The loss of resistance to dissolution with trypsin which bacterial cells undergo on heating and the greater susceptibility of heat-killed, gram-negative bacteria to lysis with proteolytic enzymes has not been adequately explained in terms of cellular structures.

On the basis of observations showing that heat-treatment resulted in rupture or damage to the walls of certain bacteria (46) it seemed probable that susceptibility of heated bacteria to lysis with proteolytic enzymes may be governed by the nature of the cell wall and the effects of heat thereon (19). Thus the walls of some gram-negative bacteria may be damaged sufficiently to expose the coagulated protoplasmic contents of the cells to the direct action of proteolytic enzymes. It is of interest to note that the cell walls constitute the

principal structural components remaining after maximum lysis of bacterial suspension with trypsin (19). This was not entirely unexpected as the isolated walls of bacteria are not lysed by trypsin.

The chemical nature of the cell walls of the gram-negative bacteria undoubtedly determines their greater susceptibility to damage on heat-treatment and subsequent ease of digestion with proteolytic enzymes. Two distinguishing characteristics of the walls of gram-negative bacteria have emerged from studies of the chemical constitution of bacterial cell walls : the walls of the gram-negative organisms contain considerably more lipid than do the walls of gram-positive bacteria and in addition their amino-acid constitution resembles more closely that of most proteins than does the amino-acid composition of walls of gram-positive bacteria (26). The presence of macromolecular components in the walls of some gram-negative organisms (47, 48) may also confer on them greater sensitivity to heat denaturation. Whether it is the high lipid content or the nature of the protein components of the cell walls of gram-negative bacteria that is responsible for their greater susceptibility to heat damage, cannot be decided upon at this stage.

Although heated gram-positive bacteria are generally more resistant to lysis with proteolytic enzymes, not all organisms are completely resistant (19, 49). This greater resistance of heat-killed, gram-positive bacteria to tryptic digestion does not appear to be a property of the coagulated protoplasm of these organisms for it has been adequately demonstrated that enzymic dissolution of the walls of heated cells greatly facilitates the bacteriolytic properties of trypsin (19). Certain heat-killed streptococci are completely resistant to digestion with trypsin (19, 38). However, incubation of group *A* streptococci with purified *Streptomyces albus* enzyme and crystalline trypsin results in lysis of the heated cells (38). Furthermore, various actinomycetes effecting lysis on *Strep. faecalis* cell-water agar (36) also produced lysed zones when grown on agar containing heat-killed group *A*, *B*, *C* or *D* streptococci (28). Thus lysis of heat-killed streptococci requires a cell-wall degrading enzyme as well as a proteolytic enzyme.

The extensive use of heat-killed bacteria in studies of the bacteriolytic substances produced by various micro-organisms has, I believe, led to much confusion when one attempts to translate reports of such activities into terms of cellular structures affected. Many of the micro-organisms secreting extracellular bacteriolytic enzymes, in particular the actinomycetes, also produce proteolytic enzymes active against heat-killed bacteria (50). Muggleton and Webb (51) fractionated the enzymes from the proteolytic system of a soil actinomyces and obtained a trypsin-type proteinase. Thus lytic zones produced by micro-organisms growing on agar media containing heat-killed bacteria may be due entirely to the secretion of proteolytic enzymes. If the production of a zone of lysis is the criterion on which a micro-organism is selected for its bacteriolytic capabilities and if in addition the relatively non-specific proteolytic systems are to be avoided, then it would be wise to refrain from the use of heat-killed bacteria and more selective methods should be adopted.

The susceptibility of bacterial cytoplasmic proteins to digestion with proteolytic enzymes is not in itself a sufficiently unique feature to make such enzymes particularly informative cytochemical reagents. The main value of the bacteriolytic properties of the proteolytic enzymes thus seems to reside not so much in the proteins they break down, but more in the nature and disposition of the structures, particles, granules, *etc.* remaining after digestion.

### *Some trends and perspectives in the enzymic analysis of bacterial cellular structure*

There is now an increasing body of information about the chemical constitution of the flagella, capsules, slime layers and cell walls of bacteria. The investigations of the action of specific enzymes on these structures has extended our understanding of the architecture of the complex bacterial surface. With the isolation of specific cellular components and the study of their degradation by enzymes, a more explicit knowledge of the molecular arrangement of their components is being gained.

Although it has been of interest to learn that capsular substances may be removed enzymically without affecting the viability of the organisms, the bacteriolytic enzymes possessing a direct action on the rigid cell-wall structures have attracted especial attention, for the outer wall is more closely associated with the ability of the cell to reproduce. Thus the cell-wall degrading enzymes have a two-fold appeal : they will facilitate the study of the molecular structure of the walls which give the bacteria their characteristic form and shape, and they will provide a means of determining the possible physiological contribution of the cell walls to various cellular functions.

It is now known for certain that the two bacteriolytic enzymes, lysozyme and the streptolytic enzyme produced by *Streptomyces albus* have a specific action on certain bacterial cell walls. There are indications that enzymes with similar functions exist for other chemically different microbial walls. A systematic search for still other systems will undoubtedly prove fruitful. The possibility that enzymes engaged in the synthesis of cell-wall structures may, under certain conditions catalyse the breakdown of wall components, appears worthy of experimental consideration. Indeed, it seems feasible that some autolytic enzymes may in fact participate in the biosynthesis of bacterial structures and that they may act as bacteriolytic enzymes when the delicate balance of cellular organization is disturbed.

Most of the known cell-wall depolymerizing enzymes are largely confined to gram-positive bacteria. A search for similar lytic systems for the walls of gram-negative organisms has so far been unsuccessful (36). The high lipid contents and the nature of the protein components of their walls may confer on the gram-negative bacteria a greater degree of resistance to enzymic lysis than that exhibited by certain gram-positive organisms. It seems likely that the components forming the rigid framework of the cell walls of gram-negative bacteria may be protected by overlying layers of other components of different chemical constitution. Thus a lytic system for the walls of these organisms may require more than one enzyme. Micro-organisms possessing a coincident

production of enzymes for specific protein, lipid and polysaccharide components of the walls of gram-negative organisms, may occur infrequently. Apart from microbial bacteriolytic enzymes, similar systems from other organisms and the potentialities of immune bacteriolysis should be investigated. The interesting observation that T2 bacteriophage « ghosts » can kill and « lyse from without » *E. coli* cells (52), would imply that bacteriophages may be a more promising source of cell-wall degrading systems for gram-negative bacteria.

The lytic enzymes are destined to play an increasingly important rôle in the integration of our knowledge of the structure and functions of the bacterial cell. The elegant use of lysozyme in dissolving the cell wall of *B. megaterium* under controlled conditions in sucrose, has provided a sub-cellular, protoplast system in which much of the structural integrity and many of the physiological properties of the intact organism are retained (1). Protoplast formation may also furnish an explanation for an earlier observation by Warren and Durso (53) that treatment of *Achromobacter fischeri* with lysozyme in the presence of 3 % sodium chloride, resulted in a morphological change from short rods to somewhat spherical bodies without any loss of bioluminescence.

With such protoplast systems, the possible contribution of the rigid outer wall to various functions of the cell may be studied and there are already exciting indications that the delicate bacterial protoplast can accomplish complex processes in the absence of an outer cell wall. There is every hope that we shall be able to extend such studies to groups of organisms other than the lysozyme-sensitive bacteria. It seems then, that our interest in bacteriolytic enzymes will not only advance our understanding of cellular structure but will inevitably broaden our knowledge of the manner in which the minute bacterial cell has condensed so many intricate functions within the confines of its outer structures.

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# The existence of a cytoplasmic membrane in the cells of 'Bacillus megaterium'

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(*Received* 23 *May* 1955)

Dr. Salton mentioned in his comprehensive review that the evidence for the existence of a cytoplasmic membrane has been less satisfactory than for other bacterial structures. I would like to describe some recent experiments on living cells that suggest the existence of a cytoplasmic membrane or at least a permeability barrier as differentiated in space from the cell wall. In this connection some experimental work on the bacterial protoplasts will also be reported.

Figure 1 shows a living cell of *Bacillus megaterium* under the microscope, suspended in Indian ink together with protoplasts of the same organism. The protoplasts were prepared by treating the bacterial cells with lysozyme in a sucrose solution (1) and were afterwards fixed in 4 % formaldehyde. The rod shaped cell consists of a central part, surrounded by an outer, somewhat brighter envelope. It can be seen from figure 2 that this envelope represents the rigid cell wall. Here cells have been squeezed between the slide and coverslip. In part of the cells the protoplasmic constituents have left the cell and have been replaced by the dark Indian ink. The bright layer remains unchanged and obviously represents the cell wall. This is particularly evident from the appearance of those bacteria which are only partly empty. Both the unchanged and the empty parts are surrounded by the same envelope.

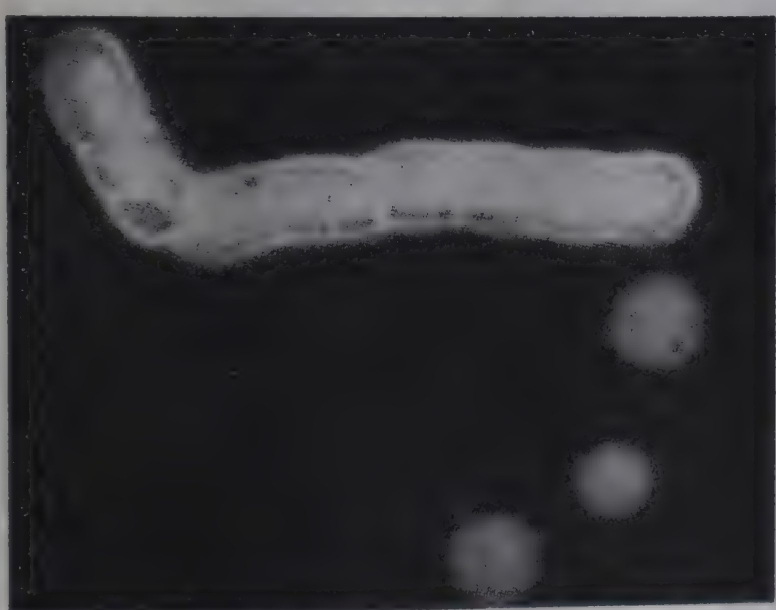

**Fig. 1. — Cells and protoplasts of *B. megaterium* in dilute Indian ink. Magnification 3800 fold.**

It can be seen in figure 1 that no bright envelope surrounds the bacterial protoplasts. This shows that the cell wall is removed by the lysozyme, leaving the protoplasm in the form of spherical bodies.

The average volume of the bacterial cell and of the included protoplasm has been determined by measuring,

**Fig. 2. — Cells of *B. megaterium*, squeezed between slide and coverslip. Medium : dilute Indian ink. Magnification 3800 fold.**

under the microscope, the length and width of a great number of bacteria and the width of the protoplasm. It has thus been found that the volume of a quantity of living bacteria, corresponding to a dry weight of 1 g., is 4.5 ml. and the volume of the included protoplasm 2.5 ml.

The permeability of resting *B. megaterium* cells to various solutes under semianaerobic conditions has been determined by adding a solution containing a known amount of the substances to be investigated to a heavy bacterial suspension. After some time the cells were precipitated by centrifugation and the concentration of the solute in the supernatant was determined.

We get the space not permeated by the solute in the final solution :

$$V_{\text{imp}} = V_1 + V_2 - (V_2 \times c/c_s)$$

where $V_1$ represents the volume of the original bacterial suspension, $V_2$ the volume of the added solution, $c$ and $c_s$ the initial and final concentration of the solute.

Similar measurements have recently been made by Mitchell and Moyle on *Micrococcus pyogenes* cells (2).

The experimental results show that under the given conditions the greater part of the bacterial body is impermeable to a number of solutes such as phosphate ions, sucrose and cozymase. In quantitative terms the impermeable volume is 2.9 ml. per g. dried bacteria, *i.e.* considerably smaller than the volume of the whole cells, 4.5 ml., but only slightly larger than the volume of the protoplasm, 2.5 ml. These facts are most easily explained by assuming a permeability barrier at the surface of the protoplasm. The difference between the morphologic-

ally determined volume of the protoplasm, 2.5 ml., and the impermeable volume, 2.9 ml., most probably represents the volume of the polypeptide and polysaccharide molecules of the cell wall.

In higher organism plasmolysis experiments conducted in a quantitative way have disclosed the existence of a semipermeable membrane around the cells. Provided the cell behaves as a perfect osmometer, the volume changes of the cell, when exposed to a heterotonic solution of a non-penetrating substance, should follow the Boyle-Van't Hoff law $PV = \text{const.}$, where $P$ represents the osmotic pressure of the medium and $V$ the volume of the cell.

In the case the cell consisted of a membrane-less gel, the volume changes would not likely follow this law.

Many experiments have shown that the law is obeyed satisfactorily if one takes into account the fact that each cell contains a dead space $b$, filled with osmoticly inert substances, such as lipids and proteins, whereas the remainder, $V - b$, holds the osmoticly transferable water. The Boyle-Van't Hoff law then takes the form $P(V - b) = \text{constant}$.

Many bacteria are plasmolysable, especially the gram-negative ones, but to the author's knowledge no quantitative measurements of the volume changes have been reported. *B. megaterium* is not plasmolysable (3) Therefore I should like to mention some recent investigations on the osmotic properties of the protoplasts of this and another *Bacillus* species.

Stähelin has described the osmotic properties of protoplasts of *Bacillus anthracis* (4). The protoplasts were obtained by suspending the bacteria in Ringer's solution. In this medium the protoplasm of many cells moves out of the cell wall and form free spherical bodies. Stähelin has been able to show that the volume changes of the protoplasts in solutions of changing osmotic pressure follow the law $P(V - b) = \text{constant}$, approximately. The author points out, however, that one has to assume a value of the quantity $b$ out of question for a bacterial cell in order to obtain a good approximation.

Also the osmotic properties of protoplasts of *B. megaterium* have now been investigated. The protoplasts were obtained by treating the cells with lysozyme in sucrose or polyethylene glycol solutions.

Table I shows that the osmotic pressure of the medium before the cell wall has been dissolved has little or no influence on the volume of the protoplasts. This volume is about half the volume of the protoplasm in the living cells. Consequently, the experimental data do not allow an estimation of the osmotic pressure inside the intact bacterial cell.

Once the protoplasts have been formed they respond to changes in the osmotic pressure of the external medium. The volume decreases when the pressure increases and *vice versa*. The plasmolysis is reversible, in polyethylene glycol solutions in a nearly quantitative way, as is shown in table II.

TABLE II

*Plasmolysis of protoplasts in PEG solutions*

| Addition of | Average volume of protoplasts (ml. × $10^{12}$) |
|---|---|
| | 2.30 ± 0.08 (*) |
| PEG | 1.59 ± 0.06 |
| Water | 2.36 ± 0.08 |
| PEG | 1.48 ± 0.06 |
| Water | 2.48 ± 0.08 |
| PEG | 1.36 ± 0.06 |

(*) Initial volume.

The average volume of the protoplasts was determined after each addition of PEG or water.

Figure 3 shows that the volume changes follow the law $P(V - b) = \text{constant}$ or $V = \text{constant}/P + b$.

The independence of the protoplast volume of the initial osmotic pressure of the medium is difficult to explain. One could assume a more or less free passage of low-molecular weight substances into and out of the protoplasts during the formation of these bodies. This explanation is, however, not very probable since no low molecular weight, ultraviolet-absorbing substances (1) or inorganic phosphate (5) are found in the medium after the bacterial cells have been treated with lysozyme in sucrose solutions.

The osmotic experiments just described indicate that the structure of the bacterial protoplasm is slightly

TABLE I

*Number and volume of protoplasts (% PEG in solution)*

| | 5.8 | 7.7 | 16.0 |
|---|---|---|---|
| Average vol. of one protoplast, ml. . . . | $(2.52 \pm 0.05) \times 10^{-12}$ | $(2.52 \pm 0.05) \times 10^{-12}$ | $(2.48 \pm 0.05) \times 10^{-12}$ |
| Number of protoplasts/g. dried whole cells | $(5.37 \pm 0.09) \times 10^{11}$ | $(5.27 \pm 0.11) \times 10^{11}$ | $(5.52 \pm 0.12) \times 10^{11}$ |
| Total volume of protoplasts, ml. g. dried whole cells . . . . . . . . . . . . . | 1.35 ± 0.04 | 1.33 ± 0.04 | 1.37 ± 0.04 |

The protoplasts were obtained by lysozyme treatment of *B. megaterium* in PEG solutions of various concentrations. Dry weight of the whole cells = 39.5 mg./ml. suspension. The error limits represent standard deviations.

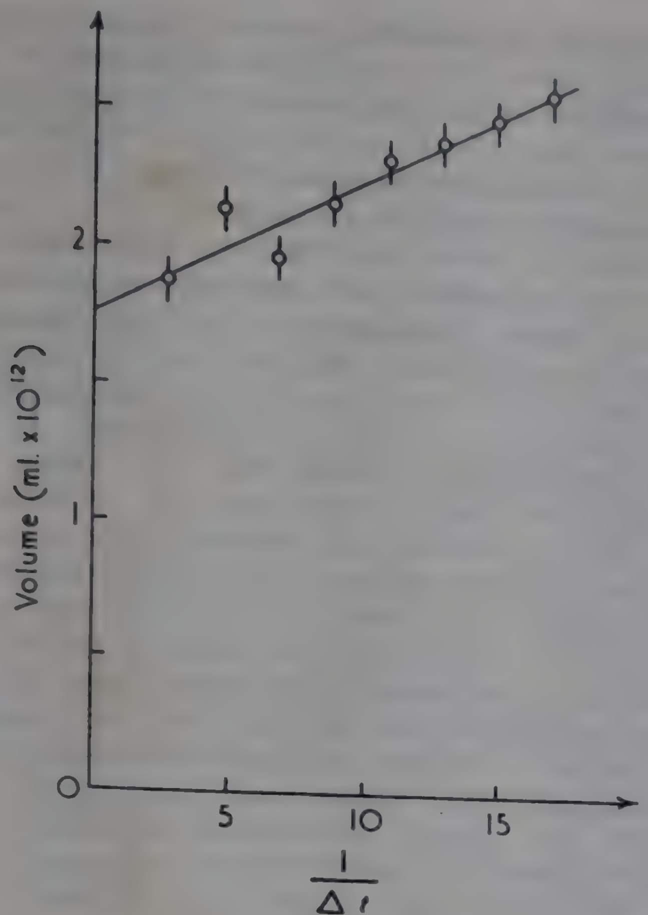


FIG. 3. — Plasmolysis of protoplasts in polyethylene glycol solutions as a function of the osmotic pressure of the medium. The volume of the plasmolysed protoplasts is plotted against the reciprocal value of the corresponding freezing point depression of the solution. The straight line is drawn by the method of least squares.

changed when the free protoplasts are formed. Hence the protoplasts cannot be regarded as strictly equivalent to the protoplasm in the cells, but some tentative conclusions concerning the structure of the living bacterium may nevertheless be justified. The validity of such conclusions is strengthened by the fact that the osmotic experiments with protoplasts and the permeability experiments with whole cells, as described above, indicate that the permeability of the two kinds of bodies is qualitatively the same in regard to sucrose, phosphate and urethan. Moreover, as has been shown earlier (1), the living cell and the protoplast have an identical endogenous respiration and oxidise glucose at the same rate.

The existence of a semipermeable membrane at the surface of the protoplasm in the living cell is strongly suggested by the fact that the volume changes of the protoplasts in heterotonic solutions follow the modified Boyle Van't Hoff law. The membrane may be very thin and soluble in lipid solvents; this would explain why a protoplasmic surface structure is not seen in electron micrographs of sectioned bacteria (6).

The volume of the osmoticly inert substances, *i.e.* the quantity $b$ in the equation $P(V - b) = \text{constant}$ has been found to represent about 35 % of the volume of the protoplasm of the living *B. megaterium* cells. A comparison with the weight and volume of the desiccated protoplasmic constituents, obtained by simple gravimetric analyses and specific volume determinations, show that the osmoticly inert substances besides high-molecular weight compounds also consist of bound water.

The remaining part of the protoplasm should then represent the 'free space' inside the cells, containing osmoticly transferable, free water and dissolved low-molecular weight substances.

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## The multiplicity of bacteriolytic agents in actinomycetin

by M. WELSCH, J. M. GHUYSEN and A. CASTERMANS

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(*Received* 26 *July* 1955)

It is evident from Dr. Salton's report that enzymes from *Streptomyces* spp. are a useful tool for the study of bacterial structures. Therefore, it is not out of place to discuss briefly, in this symposium, the nature of the lytic systems involved.

The ability of many actinomycetes to dissolve bacteria was first incidentally observed in 1921 by Lieske (17), independently re-discovered, in 1924, by Gratia and Dath (11), and studied by Gratia and his collaborators between 1924 and 1934 (13). During that period, occasional observations of the same type were made by Rosenthal, in 1925 (21), and by Borodulina in 1935 (4). In 1936, one of us started a study of the bacteriolytic activities to be found in active culture-filtrates from actinomycetes (24), to which the name actinomycetin (23) was given : the study has been going on in his laboratory

ever since. At about the same time, contributions to the subject were published by Krassilnikov and Koreniako (15) and by Kriss (16).

However, the present interest in bacteriolytic enzymes from actinomycetes developped at a later date, when three new teams of workers, enterered the field : in 1948, Webb and his collaborators (14, 20) ; in 1951, van Heyningen and his collaborators (3, 22) ; and lastly, in 1952, McCarty (18). This renewal of interest in the question seems to have been stimulated by Maxted's paper (19) showing that lysis of streptococci by *Streptomyces* enzymes is a good means of releasing streptococcal group antigens, a particular example of a general principle reported earlier by Gratia and Dath (12) and later confirmed by one of us (5).

Different organisms and techniques being used by the different workers who, in addition, are stimulated by interests of various kinds, it is very difficult to validly compare their results. In the following, we shall endeavour only to show very briefly that our own actinomycetin, that is a suitable culture-filtrate from our *Streptomyces albus* strain G, contains a number of different principles acting specifically upon given bacterial species or even strains.

At first, only two types of activity were distinguished : lysis of heat-killed bacteria on the one-hand, lysis of living gram-positive bacteria on the other hand. The typical substrate used for the study of the former activity being heat-killed *Escherichia coli*, the responsible agent was called the colilytic principle. The properties of this agent clearly show that it is a protein and an enzyme (24). A number of facts suggest that it is not identical with the many proteases and other enzymes (1, 2, 10, 25) to be found in actinomycetin and direct evidence in favour of this point was later on obtained by Ghuysen. The typical substrate used for studying the lysis of living bacteria was *Micrococcus pyogenes* var. *aureus*, and the responsible agent was therefore called the staphylolytic principle.

A number of observations show that colilytic and staphylolytic principles are distinct. The kinetics of lysis by actinomycetin follows a different course whether heat-killed *E. coli* or living *M. pyogenes* are used as a substrate (24). The conditions for the production of the two types of activity by *Streptomyces albus* G are sharply different, and so are the respective degrees of stability in culture of the two principles (7). The most suitable conditions for lysis of heat killed *E. coli* are quite different from those required for the lysis of living staphylococci : in particular, with respect to activating and inhibiting agents (7). The two principles behave quite differently with respect to absorption and elution on a variety of substrates, especially on the bacterial cells themselves (8). It was our first belief that the staphylolytic system comprized the colilytic agent plus something else, as in fact it is quite easy to obtain colilytic actinomycetin without any staphylolytic activity. But, it has now been possible to obtain separate fractions possessing only either the colilytic or the staphylolytic activity.

The staphylolytic system however is a complex one : it comprizes a lytic agent and a non lytic activator, the separation of which can be achieved by fractionnal extraction of actinomycetin with ammonium sulfate solutions of various strengths. In fact, recent observations, bearing on purification of actinomycetin by ion-exchangers, even show that the staphylolytic system might comprize three components : an activator and two other agents, each one of which behaves either as a lytic principle or as a second activator according to the staphylococcal strain used as a substrate.

It was first believed that what we called the staphylolytic system was responsible generally for the bacteriolysis of all living gram-positive bacteria sensitive to actinomycetin. However, evidence was obtained showing that the lysis of streptococci was attributable to a complex lytic system which, at least in part, is distinct from the staphylolytic system (26, 27). Next, it was shown that the lysis of pneumococci, whether heat-killed or alive, involved four different lytic agents, two of which are certainly different from either the colilytic, the staphylolytic or the streptolytic principles (9).

Lastly, it was found that a number of gram-negative rods, resistant to actinomycetin, unless they have been previously heat-killed, are dissolved by our preparation if submitted to its action at a temperature of 60° to 65° C., the rate of lysis being higher than the rate of thermal sterilization (6). Again, fractionnal extraction shows that the lytic principle here involved is different from the colilytic agent and the other lytic systems already known.

In conclusion, the bacteriolytic properties of actinomycetin from *Streptomyces albus* G are attributable to a variety of enzymes, or rather enzymatic systems, displaying each a very high degree of specificity. Work is now in progress to try and separate the many active components of actinomycetin. When this will be achieved it will be fascinating work to test their respective potential activities upon the various bacterial components that are being isolated in other laboratories, since the result of such collaborative work might well enlighten our knowledge of bacterial structures.

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# Antibiotic polypeptides

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(*Received* 23 *March* 1955)

Our present considerable interest in polypeptide antibiotics dates from a discovery of Dubos (1) made shortly before 1939 at the Rockefeller Institute. In the course of studies on bacterial antagonism with *Bacillus brevis* he noted an antibiotic effect which persisted following autolysis of the culture. Attempts to isolate the active principle led to a non-protein preparation which was recognized as being of polypeptide nature. It was called « Tyrothrycin ». In further studies by Hotchkiss and Dubos (2), tyrothrycin was easily separated by fractional crystallization into two different antibiotically active preparations which were called « Tyrocidine » and « Gramicidin ». It is now more than 16 years since the first paper on this work was published. The intervening years have seen great advances both in microbiological methods and in chemical methods for studying the structures of the principles elaborated. Although tyrocidine and gramicidin proved to be somewhat disappointing from a medical standpoint because of their toxicity, the importance of this earlier work to the development of the field of antibiotics must not be underestimated.

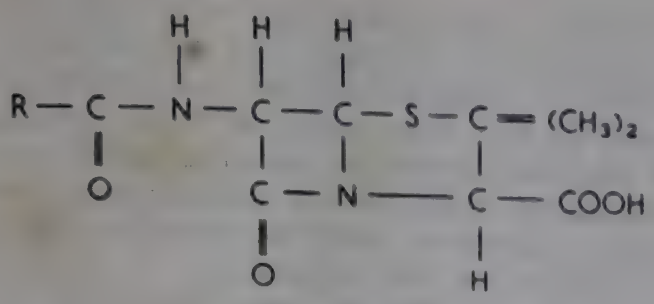
Underlying the work that has been done in the field of polypeptide antibiotics, there have been three main interests. One may be considered the microbiological interest. A second relates to the production of something of direct importance and practical use in medicine for the control of disease. A third is connected with the study of the chemistry of the substances and determination of their structure. In the present paper no attempts will be made to cover the first two interests.

Even as regards the chemistry of the polypeptide antibiotics, the significance of the study has been much more than merely that of the riddle of their structure. At the time gramicidin was discovered, there was beginning to be considerable discussion among chemists of how to go about the study of the structure of proteins. Proteins seemed hopelessly large and complicated. Gramicidin was therefore seized upon as being a simpler model of a pure substance on which to work out and test experimental methods. As other polypeptide antibiotics became available, they too were used as models for the perfection of methods useful in the broader field of protein chemistry. The development of structural concepts of most of the antibiotic peptides is therefore intimately connected with the development of the foremost methods of structural study in the protein field. These include the development of partition chromatography, ion exchange chromatography, paper chromatography (P.C.), countercurrent distribution (C.C.D.) and all the supporting techniques that make these separation techniques so effective.

In the earlier part of the era of these antibiotic substances, the approach of classical organic chemistry was dependant on fractional crystallization for deciding whether or not a substance of this type was pure. Thus gramicidin was thought to be a single pure substance because its properties did not change on repeated crystallization. Although chromatography failed to show that it was a mixture, C.C.D. (3, 4) showed that the preparation was a mixture of at least four different closely related polypeptides. This experience was soon repeated with other classes of peptides. With the proper use of C.C.D. and when it is integrated with the other techniques, it is now possible to work along the lines of classical organic chemistry whether or not crystalline substances can be obtained. In fact it is possible to proceed on a much more certain basis. We now know that crystallinity in itself offers no assurance that a given preparation is a single substance.

Up to the present time many polypeptides having antibiotic properties have been isolated. There is certainly not time in this short discussion to deal adequately with each one. Therefore a rather arbitrary choice of a limited number of main classes will be made. A discussion of these will serve to show some of the basic problems and the way these problems have been overcome. It will also serve to show the type of structure likely to be encountered in new or as yet undiscovered peptide antibiotics.

In discussing the groups chosen, it seems appropriate to speak first of the penicillins eventhough their chemistry is now familiar to everyone. They have a common structure shown in formula 1. This structure appears to be quite generally accepted. Even though penicillin G has been synthesized by du Vigneaud and collaborators (5), the correctness of formula 1 has been questioned in certain quarters. The above structure was the result of a prodigious effort by many workers (6) during the war. It can be regarded as that of a modified tripeptide. Certainly the basic purification and characte-

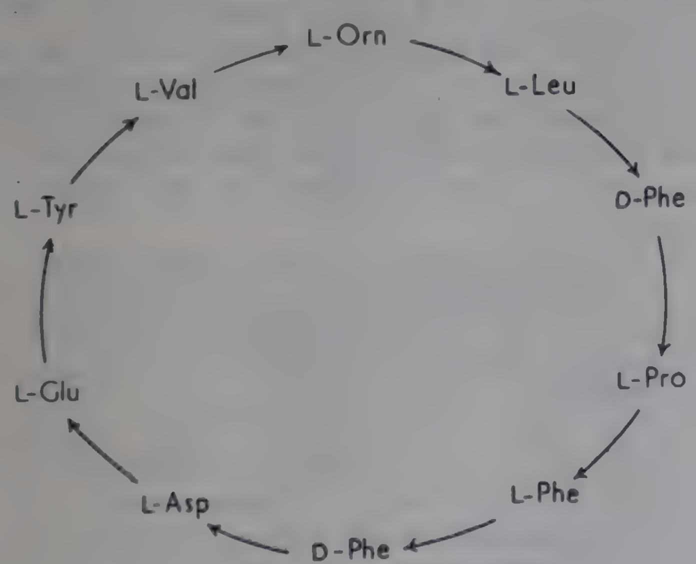


Formula 1.
R = various organic radicals.

rization would have been done with a fraction of the effort, if the separation tools available today had been available then.

Gramicidin has been resolved by C.C.D. into three peptides (4) called A, B, and C. The complete structure of none of these has been worked out. They are cyclic peptides without acidic or basic groups. The best estimate of the molecular weight is by X-ray measurement of the crystal (ref. 4, p. 73). A value 3800-4000 has been indicated. As far as is known B differs from A by the replacement of two of the tryptophan residues by phenylalanine residues. C contains at least one tyrosine residue. A and B do not contain this amino acid. All three give ethanolamine on hydrolysis, a fragment which probably comes from the breakdown (8) of a cyclic structure.

With the tyrocidine group much more progress has been made. This group has been resolved also by C.C.D. into individual peptides A, B, and C, which differ in their amino acid content (4). The molecular weight of tyrocidine A has been rigidly established by the method of partial substitution (9). Partial hydrolysis studies (10) have led to a unique sequence of amino acids as shown in formula 2. In this formula the δ-amino group of the




Formula 2.
An arrow → signifies a C to N linkage.

single ornithine furnishes the only basic group. The hydroxyl group of the tyrosine is free. Two amide groups are present which cover the extra two carboxyl groups of aspartic acid and glutamic acid. Direct evidence for every sequence was obtained. Very exhaustive fractionation attempts failed to reveal any sequences not compatible with formula 2. These results would appear to give direct evidence for a 10 amino acid residue containing 30 atoms.


Formula 3.
An arrow → signifies a C to N linkage.

Studies with tyrocidine B in collaboration with Dr. T. P. King (11) have shown that it differs from A by replacement of one of the three phenylalanine residues by a tryptophan residue. Again partial hydrolysis studies have given a clear picture of a ring structure with 30 atoms. The sequence was found to be identical with that of A, except for the substitution of an L-tryptophan residue for the L-phenylalanine residue. This indicates formula 3 to be that of B.

It would appear pertinent to point out that in these studies the isolation of both amino acids and peptides from the partial hydrolysis was done by C.C.D. with sufficient material to permit identification by classical procedures including the determination of optical activity. The latter has long been a useful property for gaining insight into the more subtle structural features. It may be that the two dextro amino acid residues are required for ready closing of the large ring.

Gramicidin-S belongs to the tyrocidine group. The crude material can be separated by C.C.D. into at least four different peptides (4). The major one wich we shall call S-A has been shown by the method of partial substitution to have a molecular weight in the range of 1100 (12). It is therefore a decapeptide. The sequence was determined by Consden, Gordon, Martin and Synge (13) in one of the earliest studies in which the problem of sequence was attacked. However, they thought the peptide was a pentapeptide since only five amino acids and five sequences were found, namely L-Val→L-Orn→L-Leu→D-Phe→L-Pro. It is quite apparent that this ring must be enlarged to twice its size and would again give a 30 membered ring. It is interesting that the sequence is identical with the sequence of half of the tyrocidine molecule even to the configuration of the phenylalanine residue.

The polymyxins (14) comprise an interesting and rather complicated class. In general the different types are formed by different strains of closely related bacteria. This group of antibiotics was discovered almost simultaneously in three different laboratories (15, 16, 17).

They are strongly basic cyclic substances all containing α, γ-diamino-butyric acid and a fatty acid attached to the periphery of the ring at some point.

TABLE I.

*Amino acid composition of the polymyxins.*

| | A | B | C | D | E | Polypeptin | Circulin |
|---|---|---|---|---|---|---|---|
| Leucine . . | D | 1L | | 1D | D | 2L | 1D |
| Phenylalanine . . . | | 1D | L | | | 1D | |
| Threonine . | L | 2L | L | 3L | L | 1L | 1L |
| Serine. . . | | | | 1D | | | |
| α.γ-diamino-butyric acid | L et D,L | 5L 1D | L | 5L | L | 3L | 5L |
| Valine . . | | | | | | 1D | |
| *Iso*leucine . | | | | | | 1L | |

Table I gives the amino acid composition of five of the original polymyxins and, in addition, two newer antibiotic polypeptides which obviously belong in this class by virtue of the fragments they give on hydrolysis.

Bell *et al.* (18) carried out composition and structural studies on a preparation of polymyxin D. Although there was experimental evidence that the preparation was not entirely pure, it seems quite clear that this peptide forms a 30 membered ring with its 10 amino acid residues. Polymyxin $B_1$ (19) was found to have a ring of different size, and with a different amino acid spectrum. It is interesting to note that polypeptin (20) can contain only 27 while circulin (21) can contain only 21 atoms.

Most of the work with the polymyxins was done before C.C.D. had reached its present state of effectiveness. It therefore appeared of interest to take a preparation of a polymyxin and see if it would satisfy the criterion of purity at present available. Accordingly a sample of B was studied in the author's laboratory by Dr. Hausmann (19). This resulted in the separation of two polymyxins, $B_1$ and $B_2$. $B_1$ was found to contain the optically active *iso*pelargonic acid found in all the polymyxins except polypeptin. $B_2$ was found to contain a similar acid but with one carbon atom less. It would not be surprising to see this complication turn up in all the other polymyxins. The acid in polypeptin is of interest. It is probably a hydroxyheptanoic acid which may lose water easily to give an unsaturated acid under certain conditions.

The actinomycins comprise an interesting and complicated group of natural products with a molecular weight in the neighborhood of 1200. They were discovered by Waksman and Woodruff (22) in 1940 during an investigation of *Actinomycetes*. The field is now being rapidly exploited by Brockmann and his collaborators (23). With these substances paper chromatography and C.C.D. are proving to be the effective separation tools. Other methods have failed to give clear cut separations.

Like in the case of the polymyxins the different types are produced by different strains of the organism. However, C.C.D. has shown that each type contains a spectrum of different chemical individuals which differ quantitatively or qualitatively by their amino acid content. Table II will show some of these differences as far as the peptide portion of the molecule is concerned. Acide from amino acids all the actinomycins contain a chromophoric polycyclic quinone which is identical in at least a number of the antibiotics. It is thought to be a dihydroxydimethylquinone derivative of acridine (24). The actinomycins do not contain amino or carboxyl groups. It is therefore likely that they are cyclic polypeptides.

TABLE II.

*Amino acid composition of the actinomycins*

| Actinomycin | L-Threonine | Sarcosine | L-Proline | D-Valine | L-N-methyl valine | D-*Allo*-*iso*-leucine |
|---|---|---|---|---|---|---|
| $C_1$ | 0.9 | 1.5 | 1.7 | 1.9 | 1.7 | — |
| $C_2$ | 1.4 | 1.4 | 1.9 | 0.9 | 1.9 | 0.9 |
| $C_3$ | 1.2 | 1.4 | 2.1 | — | 2.0 | 2.0 |
| $X_1$ | 0.9 | 1.9 | 2.1 | 1.9 | 1.9 | — |
| $X_2$ | 0.7 | 2.1 | 1.1 | 2.1 | 2.1 | — |
| $X_3$ | | | | | | |
| $X_4$ | | | | | | |
| $I_0$ | + | + | + | + | + | — |
| $I_1$ | + | + | + | + | + | |
| $I_2$ | | | | | | |
| $I_3$ | | | | | | |

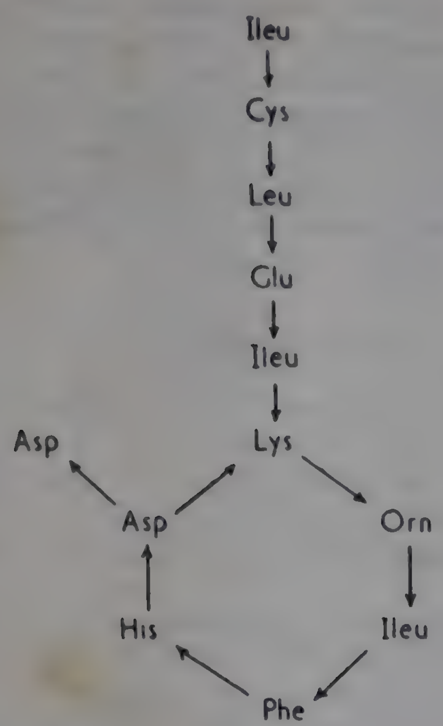
The bacitracin polypeptides discovered by Johnson and Melaney (25) in 1943 are especially interesting from many standpoints. This family of polypeptides is produced by a strain of *Bacillus licheniformis*. Thus far C.C.D. has been the best tool for separating the individual members (26, 27, 28). Chromatography (29) has given a separation of bacitracin A. Efforts toward the complete elucidation of the structure of the most abundant member of the family, A, are now nearing completion.

The molecular weight has been established by the method of partial substitution as approximating 1500 (30). Quantitative amino acid analyses and isolation of the acids following hydrolysis (31, 32) have resulted in a formula. L-$Ileu_2$ D-Phe L-His D,L-$Asp_2$ D-Glu L-Cys L-Lys D-Orn with the *iso*leucine and phenylalanine being partly racemized. This composition is in agreement with the overall analytical composition and therefore accounts for the whole molecule (32).

Partial hydrolysis studies have been carried out independently in four different laboratories (33, 34, 35, 35*a*). Two of these, (33 and 34), are in complete agreement (*) and indicate a sequence shown in formula 4. This sequence, however, by no means gives the structure of bacitracin A.

The arrangement around the sulfur is interesting. Bacitracin does not give a test for sulfhydryl, yet can-

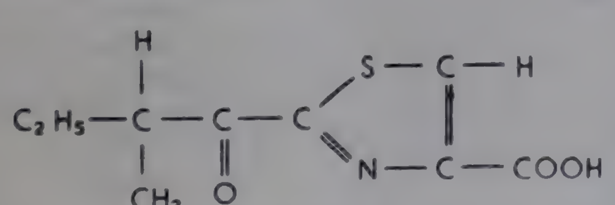
(*) Dr. Porath has written that his later work is in agreement with our sequence.



Formula 4.

not contain a disulfide linkage. The sulfur must therefore be joined to some second position in the peptide chain. On the basis of this, the relative stability, etc., Newton and Abraham (28) suggested a thiazoline ring. Proof that this is indeed so, however, has required considerable experimental work. On standing in solution at pH 7 or slightly higher bacitracin A loses nitrogen as ammonia to give bacitracin F. During this change the ultraviolet absorption is shifted from the region of 255 mμ to 290 mμ (27), indicating further double bond conjugation.

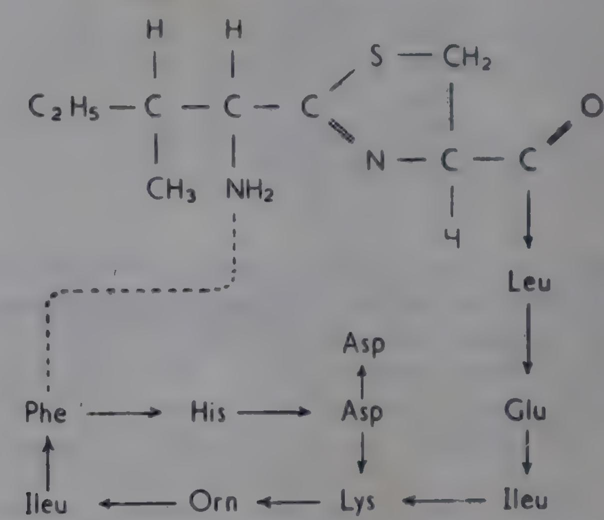
After hydrolysis of F the single sulfur has been found to emerge in a fragment which crystallized readily and had the composition $C_9H_{11}O_3NS$ (36). The fragment was an acid with no basic properties. The third oxygen was present as a keto group. Its structural formula is that shown in formula 5. The emergence of a thiazole is best explained on the basis of a thiazoline ring being present in bacitracin A.



Formula 5.

Bacitracin A gives 0.5 mole of *allo-iso*leucine on hydrolysis. This cannot be found after hydrolysis of F, tri-DNP-bacitracin A or A desulfurized with Raney nickel. Therefore this *iso*leucine is the one which has an amino group capable of reacting with the Sanger reagent and is the one which loses ammonia during the transformation to F. The thiazoline structure shown in formula 6 would be expected to give a partly or completely racemized *iso*leucine residue on hydrolysis. A further indication of the above structure is the finding (36) that following desulfurization with Raney nickel and hydrolysis, all the amino acids are found except cysteine and *allo-iso*leucine. Only a small yield of alanine was found.

From the extensive partial hydrolysis studies all the sequences found could be rationalized by the order given in formula 6 except one: Phe→Ileu→Cys→Leu. This one raises an interesting point for speculation. On the basis of published work concerning thio acids and esters, it would be expected that the linkages around the sulfur would be so-called « energy rich » bonds. That this is so is shown by the ability to undergo transformation so easily to the F type as discussed.



Formula 6.

It appears highly likely that the carbonyl of the phenylalanine is in some way involved in the grouping and following hydrolysis a small part of the phenylalanine emerges attached to the reactive amino group of the *iso*leucine. The fact that on total hydrolysis the phenylalanine emerges largely racemized indicates some unusual arrangement at this point in the chain. The dashed line in formula 6 is inserted to indicate this state of affairs.

Bacitracin B has been found to contain all the amino acids present in A and in addition a valine residue. It shows the same ultra-violet absorption spectrum and has the same number of aminogroups. When tri-DNP-bacitracin B is hydrolyzed, the expected δ-DNP-ornithine and DNP-imhistidine are found but unexpectedly about one third mole each of DNP-*iso*leucine and DNP-valine. Under these conditions bacitracin A gives a poor yield, 25 % or less, of DNP-*iso*leucine. The exact way the extra valine residue is connected to the molecule is not known but it appears to be part of the labile grouping associated with the thiazoline.

Another series of peptides, called the Licheniformins, are produced by various strains of *Bacillus licheniformis*. These substances were discovered in 1945 by Callow and D'Arcy Hart (37). The crude antibiotic can be separated by C.C.D. into three distinctly different antibiotic peptides. It might be expected that they would be similar to the bacitracins but quantitative amino acid analyses show otherwise (38). Sulfur is absent. Their molecular weight approximates 4100. Table III shows the compositions of A and B. C contains glutamic acid in addition to the residues found in A and B. None of the three contain a terminal N group and all

are presumably cyclic peptides. At least one D amino acid is indicated by D amino acid oxidase.

TABLE III.

*Amino acid composition of licheniformins A and B.*

| Amino acid | A | B |
|---|---|---|
| Asp | 1 | 1 |
| Gly | 7 | 7 |
| Ser | 3 | 3 |
| Pro | 2 | 2 |
| Arg | 6 | 6 |
| Phe | 2 | 2 |
| Val | 2 | 2 |
| Lys | 12 | 12 |

The nisins are still another group of larger molecular weight peptide antibiotics. These antibiotics were discovered by Mattick and Hirsch (39) in 1944 in a culture of a strain of *Streptococcus lactis*. The group was separated by Berridge, Newton and Abraham (40) into four different polypeptides, A, B, C, and D when studied by C.C.D. A, B, and C contain leucine and/or *iso*-leucine, valine, alanine, glycine, proline, aspartic acid, histidine, lysine, methionine, lanthionine and a structural isomer of cystathionine. D contains glutamic acid but no valine or methionine.

The nisins are similar to subtilin, an antibiotic produced by a strain of *Bacillus subtilis* (41). Although subtilin contains a different amino acid spectrum (42) it contains lanthionine and the isomer of cystathionine (43). Thirteen amino acids are present. The molecular weight is thought to approximate 3400. Overall analytical figures indicate several rings to be present.

This has been a short survey of certain arbitrarily selected groups of polypeptide antibiotics. Many others less well characterized could be mentioned. Those discussed range in size from tri-peptides to others containing 35 residues. The latter is nearly the size of the insulin proteins which are now known to have molecular weights slightly less than 6000 (44).

All of these substances are cyclic peptides with unique linkages characteristic of each group or family. The individual members within a family have such similar properties that a difficult separation problem is presented. This problem has been solved most successfully by the use of C.C.D. supported by paper chromatography, ion exchange chromatography, and zone electrophoresis. It seems worthwhile pointing out that while the data discussed in this paper were being gathered in many laboratories, the technique of C.C.D. was improved from that employing a hand operated 25 tubes steel apparatus to a fully automatic all glass apparatus containing 420 tubes (45). Separations up to 10 000 transfers (46) have been accomplished in equipment of this type. Much has been learned regarding the compounding of systems for the separation of natural products.

Finally some rather preliminary insight into the nature of certain naturally occurring peptide bond structures has been obtained. The structure of a naturally occurring polypeptide has by no means been determined when its amino acid sequence has been worked out. The bacitracin studies emphasize the fact that hydrolytic procedures may well obscure the more subtle features of peptide structure. The development of methods which will be capable of revealing such features wherever they may be found presents a real challenge to the ingenuity of the organic chemist.

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## Observations on some antibacterial polypeptides

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(*Received* 24 *July* 1955)

Dr. Craig's stimulating account of the antibacterial polypeptides has illustrated the way in which progress in the study of peptide structure has been conditioned by the discovery of new methods. We are indebted to Craig and his colleagues for a method of counter-current distribution which has facilitated both the separation of polypeptides and the determination of their molecular weights. An attractive feature of the method is the ease with which it can be used on a preparative scale. It is worth noting that Hausmann, Weisiger and Craig (1) have isolated products of the partial hydrolysis of polypeptides in a form which has enabled their elementary composition to be checked. This places the conclusions they have reached about amino acid sequences on a foundation of enviable solidity; one feels that they are not only interesting, but also very likely to be true.

Among the substances discussed in this Symposium, three families of peptides (penicillin, bacitracin and nisin) happen to have occupied a good deal of the time of my colleagues, Newton and Lockhart, and of myself. I propose here to confine my remarks to the penicillin and bacitracin family.

Dr. Craig rightly remarked that the early work on penicillin would have been very much easier if the separation tools that are now at our disposal had been available then. We have recently been able to use some of these tools to isolate a new type of penicillin which is more difficult to purify than those members of the family that were first discovered. This substance, which is produced by a species of *Cephalosporium* from Sardinia, has been called cephalosporin N. It yields D-α-aminoadipic acid on hydrolysis and appears to have the following structure (figure 1, ref. 2).

Cephalosporin N is as unstable as the common penicillins in dilute acid or alkali and is also extremely hydrophilic. It could be subjected to counter-current distribution in the region of neutrality, however, by using a solvent system composed of water, phenol, and a tertiary base such as collidine. The base acted as a carrier, as well as a buffer, and greatly increased the partition coefficient of the acidic antibiotic in favour of the phenol phase (3).

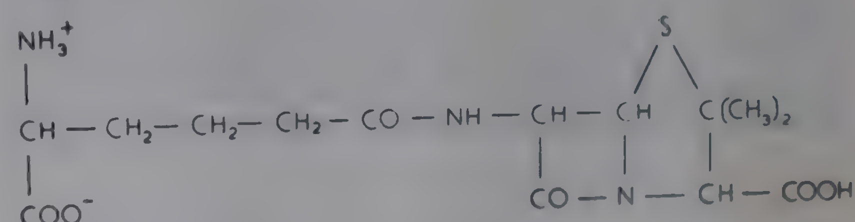

Fig. 1.

Cephalosporin N is sensitive to penicillinase, but it is strikingly different from the common penicillins in its antibacterial activity, a fact which must be attributed, as must its hydrophilic character, to the presence of an amino group in the side chain. Acylation of the amino group results in an increase in activity against gram-positive organisms and a very large decrease in activity against gram-negative organisms.

Two years ago, we suggested (4) that synnematin, an antibiotic which had been reported in the U. S. A. to be produced by certain species of *Cephalosporium* but had not been characterized chemically, might be the same substance as cephalosporin N. An exchange and direct comparison of different preparations has now produced strong evidence that cephalosporin N and synnematin B are, in fact, identical. Synnematin B has been used successfully for the treatment of typhoid fever in man (5).

The Sardinian cephalosporium also produces another hydrophilic antibiotic which we have called cephalosporin C and isolated as a crystalline sodium salt (6). Cephalosporin C contains sulphur and its provisional molecular formula only differs from that assigned to cephalosporin N in having two more carbon atoms and two more oxygen atoms. Like cephalosporin N, it yields D-α-aminoadipic acid on hydrolysis and it shows a similar activity against a variety of gram-positive and gram-negative bacteria.

This substance has excited our curiosity because it behaves like a penicillin in some respects, but not in others. For example, it shows an absorption band in the infra red region, at 5.61 μ, which is also shown by the penicillins and has been attributed to the >C=0 of the thiazolidine β-lactam ring system (7), but it is relatively stable to acid and does not undergo the penicillin-penillic acid rearrangement. It does not yield penicillamine on acid hydrolysis, but it does yield valine, which contains the carbon skeleton of penicillamine, when hydrolysis is preceded by hydrogenolysis with Raney nickel. It is not sensitive to penicillinase and has an inhibitory effect on the activity of the enzyme. Nevertheless, it has been found by Pollock (8) to induce the formation of penicillinase by cultures of *Bacillus cereus* or *Bacillus subtilis*; in fact its maximum inducing rate is four times as great as that of benzylpenicillin. Moreover, there is evidence that it competes with penicillin for reaction with the inducing centre of the bacterial cell. In view of the increasing importance of infections caused by penicillinase-producing staphylococci, it may be that these properties will have more that theoretical interest.

Florey and his colleagues (9) have shown that cephalosporin C, like cephalosporin N and benzylpenicillin, has a rapid bactericidal action on growing cultures of streptococci and staphylococci. The activity of cephalosporin C against most bacteria that do not produce penicillinase is considerably less than that of cephalosporin N. However, its toxicity is so low that a dose of 100 mg. may be given intravenously to a 20 g. mouse without noticeable ill-effect. When administered subcutaneously in appropriate amounts it has been found to afford complete protection to mice infected with at least $10^6$ lethal doses of haemolytic streptococci.

Our work on cephalosporin C has only just begun, but the facts already uncovered are sufficiently interesting to make us feel that this substance merits a detailed investigation.

Now let me turn to bacitracin. I should perhaps recall how we came to enter a field in which Craig and his colleagues have been making such notable progress. In 1947, we began work on a family of antibacterial polypeptides produced by a strain of *Bacillus licheniformis* (A5) which had been brought from Chile. The peptide family was named ayfivin (10). At that time there was some doubt whether bacitracin was a polypeptide (11) and we had no reason to think that it was related to ayfivin. In 1948, however, this situation was changed by the appearance of a paper by Barry, Gregory and Craig (12) in which some of the characteristic chemical properties of bacitracin were clearly stated for the first time. We later showed that ayfivin and bacitracin were in fact identical (13) and the name ayfivin, which was second in the field, was abandoned.

Among the most interesting problems which confronted those working on the structure of bacitracin A was the nature of the N-terminal and the sulphur- containing residues. The original suggestions that the former was a leucine or *iso*leucine residue and that the latter contained a thiazoline ring (14) now seem very likely to be true (15). I should like to make a few additions to the interesting evidence presented by Dr. Craig on these questions.

The presence of a thiazoline ring was first postulated to account for the ready liberation of a free thiol group in hot dilute acid and for certain properties of the product obtained by hydrogenolysis of bacitracin A with Raney nickel. This product contained an N-terminal alanine residue and an amino alcohol was shown to be present among the amino acids formed on hydrolysis. Further

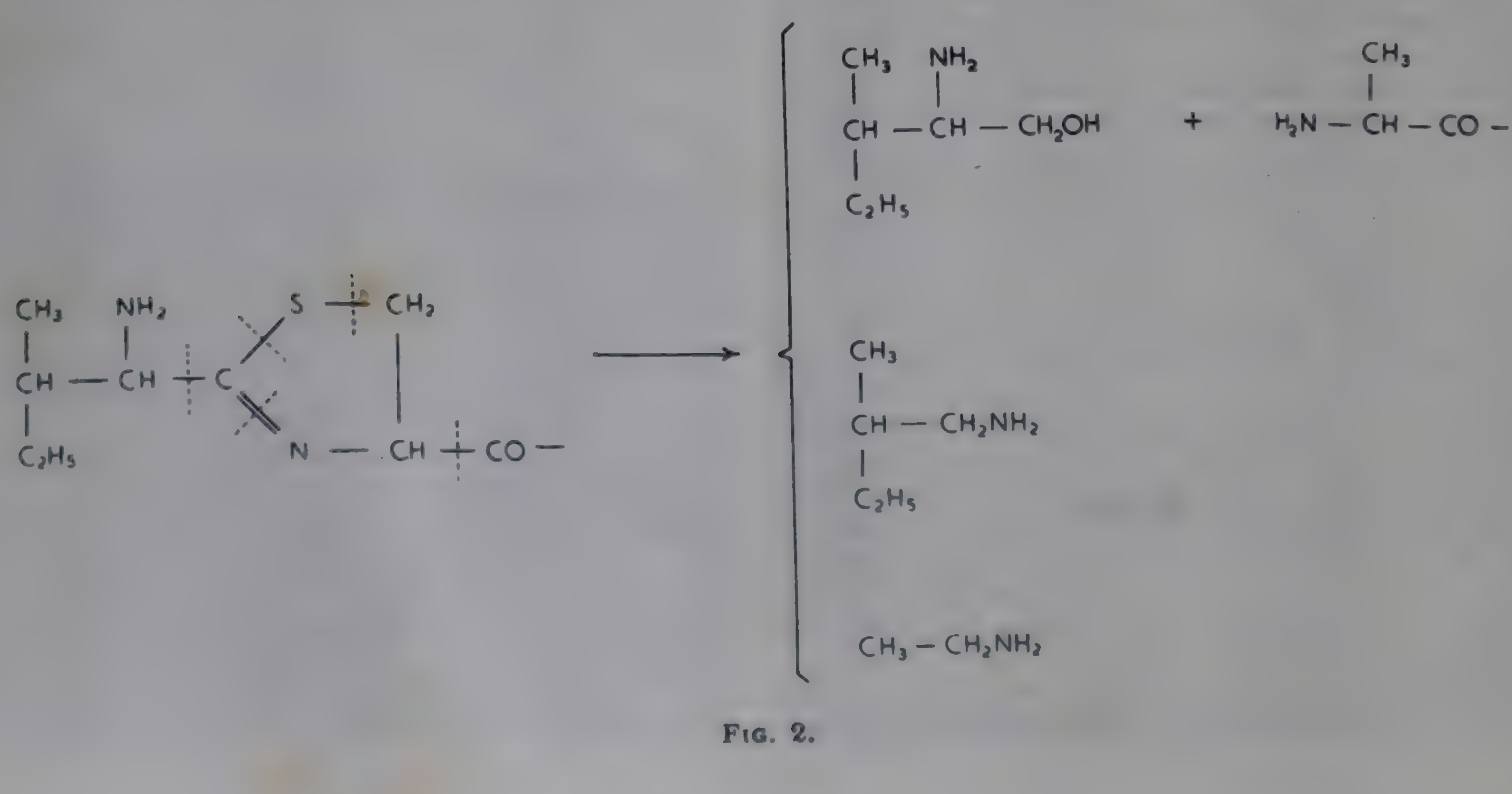

FIG. 2.

work (16) has now proved that the amino alcohol is *iso*leucinol and also that it is present in the free state before the product of hydrogenolysis is hydrolysed with acid. It has also been shown that the *iso*leucinol is accompanied by at least two other volatile bases, 1-amino-2-methyl-n-butane and ethylamine. All these products can be derived from a structure in which the carboxyl group of an *iso*leucine residue is condensed with a cysteine residue to form a thiazoline ring. The liberation of *iso*leucinol and 1-amino-2-methylbutane in the free state on hydrogenolysis shows that the *iso*leucine residue is N-terminal. The reactions now known to occur when bacitracin A is treated with Raney nickel can be illustrated by the following scheme, the dotted lines showing the bonds broken in the original structure. The breaking of carbon-carbon bonds is known to occur during the hydrogenolysis of penicillin (17).

It is the N-terminal *iso*leucine residue which is responsible for the presence of half a molecule of *alloiso*leucine in hydrolysates of bacitracin A, because no *alloiso*leucine is formed if hydrolysis is preceded by deamination with nitrous acid (18). Support has been obtained for the suggestion of Hausmann, Weisiger and Craig (1) that the *alloiso*leucine is derived from *iso*leucine by racemization. The *iso*leucinol formed on hydrogenolysis was re-oxidized to *iso*leucine. Analysis of the latter on a column of Dowex-50 showed that it contained about 66 % of *iso*leucine and 33 % of *alloiso*leucine (16). It seems likely that racemization is facilitated by the opportunity for resonance in the anion resulting from the ionization of a hydrogen on the α-carbon atom.

It is quite possible that the detailed structure of bacitracin A still holds some surprises, and it is certain that more must be learned about the chemical reactivity of the N-terminal portion of the molecule before some of the most interesting properties of this substance can be understood. Recently Calvin (19) has suggested that glutathione can exist as a normal tripeptide in equilibrium with a structure containing a thiazoline ring. With this exception, the thiazoline ring system in the structure proposed for bacitracin A is so far unique in peptide chemistry. Whether there are thiazolines in proteins, awaiting discovery when methods delicate enough to find them are used, is a question raised by Linderstrom-Lang and Jacobsen (20) in 1941 which still remains unanswered.

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## The mode of action of polymyxin


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(*Received* 12 *July* 1955)

In the fifteen years since Hotchkiss and Dubos (1) reported the isolation of gramicidin and tyrocidin more than twenty polypeptide antibiotics have been isolated and as we have seen from Dr. Craig's report the chemistry of a number of these has been studied in detail. Dr. Craig has pointed out that research in the field of polypeptide antibiotics has followed three main lines : microbiological, medical and chemical. Up to the present time microbiological studies, and in particular studies of the mode of action of antibiotics, have lagged far behind studies of their chemical constitution and in only a few cases have we any detailed knowledge of the nature of their antibacterial activity. Ultimately it will be possible to integrate the results of each of these lines of research and so obtain a complete picture of the inter-relationships between chemical constitution, mode of action and toxicity of antibiotics; although we are still far from this goal I would like to add to the mass of chemical data the results of some microbiological studies which have given an indication of the mode of action

of the polymyxin group of peptides and the relationship between their structure and antibacterial activity.

Aqueous solutions of polymyxin are markedly surface active and the addition of a bactericidal concentration to suspensions of sensitive bacteria results in a rapid release of soluble constituents from the cells (2); in this respect polymyxin resembles numerous other surface active agents. Various explanations have been put forward to account for the bactericidal action of surface active agents but at the present it seems that the bulk of the experimental evidence favours the original hypothesis of Baker, Harrison and Miller (3) who suggested that these compounds combine with and disorganise structures of the bacterial cell which are responsible for the maintenance of the osmotic equilibrium of the cell. Although this hypothesis was proposed more than fifteen years ago the recent use of a fluorescent derivative of polymyxin (4) has provided the first direct demonstration that such substances do combine specifically with a membrane underlying the bacterial cell wall.

A fluorescent derivative of polymyxin was prepared by coupling 1-dimethylaminonaphthalene-5-sulphonyl chloride with the γ-amino groups of α,γ-diaminobutyric acid in the polymyxin molecule, limiting amounts of the sulphonyl compound were used so that the majority of polymyxin molecules carried only one naphthalene group. Fluorescence microscopy showed that 1-dimethylaminonaphthalene-5-sulphonamido-polymyxin (DANSP) was readily absorbed by polymyxin sensitive bacteria and in growth tests it was found to have approximately the same bactericidal activity as the untreated antibiotic. The distribution of DANSP in a number of polymyxin sensitive organisms has been studied (4); cells treated with a bactericidal concentration of the derivative were disrupted by mechanical agitation, fractionated by differential centrifugation and the intensity of fluorescence of the various fractions measured. Examination of *Bacillus megaterium*, *Sarcina lutea* and *Micrococcus lysodeikticus* in this way showed that more than 90 % of the fluorescent conjugate was associated with a small particle fraction sedimented at 100 000 *g* and less than 10 % with the cell walls. This distribution was constant for a range of concentrations of DANSP, the highest concentration tested being more than four times the bactericidal level. When cell walls alone were treated with DANSP they absorbed up to 270 μg./mg. dry weight walls, this was not removed by repeated washing in distilled water but incubation with the small particle fraction from the cells resulted in more than 90 % being removed from the walls and taken up by the small particles. The recent work of Weibull (5) has provided a particularly promising means of studying the action of surface active compounds on the cell membrane and has also provided some evidence that the small particles obtained by mechanical or sonic disintegration of certain bacteria may be derived from a membrane underlying the cell wall. Weibull (5) has shown that controlled lysozyme treatment of *Bacillus megaterium* results in the depolymerization of the cell walls the rest of the cell remaining as an intact structural unit, a spherical protoplast, bounded by a lysozyme resistant membrane. This technique has been used to study the distribution of DANSP in *B. megaterium* (4); fluorescence microscopy of DANSP-treated *B. megaterium* before and after lysozyme treatment has shown that the fluorescent compound is associated with the protoplast membrane. After treatment of fluorescent protoplasts in a sonic oscillator all the DANSP was found to be associated with the small particles sedimented by centrifugation at 100 000 *g*. Thus it seems likely that the small particles obtained by mechanical disintegration of whole cells and which have a high affinity for DANSP may originate from a membrane underlying the cell wall; Mitchell and Moyle (6) isolated a similar fraction from *Staphylococcus aureus*, they found that these particles contained a high proportion of phospholipid and suggested that they may be derived from the lipid layer described by Burdon (7) which lies inside the cell wall and is readily stained by Sudan Black.

The chemical nature of the polymyxin-binding component of the cell membrane is as yet unknown but there is some evidence to suggest that it is a phospholipid; Bliss, Chandler and Schoenbach (8) observed that phospholipids of the lecithin type antagonise the antibacterial activity of polymyxin, Latterade and Macheboeuf (9) described the formation of water insoluble polymyxin-phospholipid complexes and Newton (10) showed from a study of the competition between certain cations and polymyxin molecules for sites on bacterial cells that the polymyxin-combining loci of the cells had the properties of polyphosphates. More recently Few (11) has studied the interaction of polymyxin with bacterial and other lipids using the monolayer technique and has obtained evidence of electrostatic bonding between polymyxin and ionised phosphate groups.

From the results that have been outlined briefly we can now draw a fairly clear picture of the mode of action of the polymyxins; the bactericidal activity of these polypeptides can be adequately explained in terms of a disorganisation of a cell membrane or barrier which controls the osmotic equilibrium of the cells, this disorganisation may be the result of a decrease in interfacial tension at the membrane produced by the combination of the antibiotic with the ionized phosphate groups of phospholipid components of the membrane. It is also likely that the penetration of the hydrophobic 6-methyl-octan-1-oic acid side chain of the polymyxin molecule into the lipid of this membrane may cause further alteration of its osmotic properties.

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# Biological nitrogen fixation

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(*Received* 14 *April* 1955)

### *The significance of $N_2$-fixation for the economy of man and nature*

Biological nitrogen fixation is a fundamental phenomenon in the maintenance of life. All the nitrogen in the world found in different nitrogenous compounds has its origin in the atmosphere. Apparently only a small portion of the fixed nitrogen results from the nitrogen fixation brought about by photochemical reactions or electric discharges in the atmosphere. In Finland, the yearly rainfall per hectare contains about 3 kg. of combined nitrogen, about 1 kg. of it being nitrate nitrogen and the rest mainly ammonium nitrogen. The latter originates, either entirely or at least partly, from the ammonia evaporated from the soil. $N_2O$ formed in denitrification may be oxidized to higher nitrogen oxides in the upper, ozone, layers of the atmosphere, wherefore part even of the nitrate may originate from the nitrogen compounds of the soil (1). $N_2$-fixation in the atmosphere is accordingly very weak. The large amounts of ammonium and nitrate nitrogen observed in Central Europe (2) in the rain water are apparently mostly due to industry.

Intensified cultivation and increasing crops require great quantities of nitrogen nutrition, and the production of nitrogen fertilizers by industrial nitrogen fixation increases steadily. For the time being perhaps only two or three % of the nitrogen contained in the annually harvested crops of the world originate, however, from nitrogen fertilizers produced by the industry. The main part of the nitrogen of plants is still a product of biological nitrogen fixation or derived from the nitrogen reserves of the soil. In the latter case we have in the long run to do with robbing the soil of its fertility, while effective cultivation of legumes is an excellent method for the preservation of the fertility of soils. There is no method of preserving the humus and nitrogen content of the soil more effective than the cultivating of legume rich leys, especially leys of clover and blue lucerne (alfalfa). Neither is there a more effective and economical way of producing proteins for the animals. The significance of leguminous plants has therefore by no means diminished in the face of the tremendous development of the manufacture of nitrogen fertilizers. From the point of view of the world economy of energy an effective cultivation of legumes, and thus the utilization of biological nitrogen fixation, is of greatest importance.

The nitrogen fixation taking place in the root nodules of legumes is, under favourable conditions, efficient for the maximal growth of legumes. Even in a country so far up in the north as Finland a good red-clover sward fixes 200-300 kg. of $N_2$ per ha. in one growth period, pea about 100 kg. In countries where summer is longer and weather favourable 400-500 kg. of $N_2$ may be fixed in a red-clover sward, and in a blue-lucerne sward apparently even more. These amounts exceed many times over the amounts of nitrogen in the fertilizers used in very effective agricultural production, even for plants yielding the largest crops (*e.g.* sugar beet, fodder sugar beet, *etc.*).

The amounts of nitrogen fixed by free living nitrogen fixing bacteria, such as anaerobic *Clostridium* and aerobic *Azotobacter*, are much smaller. Real information about the amounts of nitrogen thus fixed is lacking, but it is obvious that they vary greatly in different soils, possibly from a few kgs. to 20-30 kg./ha. In the naturally acid soils of Finland, *Clostridium* is apparently the most important free living nitrogen fixer both in forest and cultivated soils.

In 1933 I bought a farm in order to find out how effective agricultural production with milk as chief product, and with grain, potatoes, *etc.*, as quantitatively secondary products could be maintained without any industrial nitrogen fertilizers and concentrates rich in protein. This programme has now been carried through for 22 years. Atmospheric nitrogen has been fixed chiefly in clover-rich leys and pastures (calc. 200-250 kg. of $N_2$ per year and hectare). Effective utilization of dung and urine has, of course, also been accomplished. The cultivated area is 38 ha. The yields per hectare have been 3000-4000 kg. of oat, 2000-3000 kg. of summer wheat, 17 000-25 000 kg. of potatoes and 35 000-45 000 kg. of fodder sugar beet. The live-stock of the farm comprises at the present 25 dairy cows and about 20 heifers and calves. The annual production per cow is about 4000 kg. of milk and 185-190 kg. of butter fat (3). The production of the farm can thus be regarded as very high for Finnish conditions. Soil analyses show that the humus and nitrogen contents of the soil are high and that they are well maintained under self-supporting nitrogen management.

### *Mechanism of biological $N_2$-fixation*

The activation of $N_2$ and the formation of the first nitrogen compound resulting from it are to be regarded as $N_2$-fixation reaction proper. Knowledge of the later stages of the reaction is of importance in this connection only in case conclusions can be drawn about the mechanism on the basis of them.

In order to obtain a reliable notion of the mechanism of $N_2$-fixation we have to know the enzyme, or enzymes, acting in the fixation reaction. Without this knowledge we are dependent on more or less justified hypotheses. Unfortunately our knowledge about the enzymes acting in nitrogen fixation is very deficient. Since the heavy metal enzymes as a rule act in the most important reactions of the metabolism, and since this seems to be the case also in regard to nitrogen fixation, it is necessary to pay special attention to the activating effect of heavy metals on $N_2$-fixation as well as to inhibition experiments.

Bortels (4) was the first to call attention to the activating effect of molybdenum on $N_2$-fixation by *Azotobacter*. He also noticed a positive influence of molybdenum on the growth of *Azotobacter* with nitrate. Later on many others have attempted to find out if the influence of molybdenum is specific for nitrogen fixation or not. On the basis of their results many investigators have come to the conclusion that molybdenum is specifically involved in $N_2$-fixation, as well regarding the free living nitrogen fixers as the root nodules of leguminous plants. The positive influence of molybdenum on the assimilation of nitrate has been found to be relatively weak, and the optimal molybdenum concentration different when $N_2$ or nitrate has been the source of nitrogen. Practically no effect of molybdenum was found in nutrient solutions with ammonium nitrogen. There are, however, discrepancies in the results obtained by different investigators which may depend on (1) the fact that the organisms used are not made poor in molybdenum before the experiment proper (2), on the fact that the molybdenum concentration in the nutrient solution has not been determined before the addition of molybdenum salt, and (3) on the fact that the organism has not been adopted to use nitrate.

In our laboratory Mrs. Lundbom, Miss Ettala, and I have reinvestigated the problem and determined the influence of molybdenum and vanadium on $N_2$-fixation by *Azotobacter vinelandii* and *Clostridium butyricum* as well as on the assimilation of nitrate and ammonium nitrogen. In experiments with *A. vinelandii* the basal nutrient solution contained 0.000003 % Mo. The curve showing the dependence of growth with $N_2$ and nitrate nitrogen on the concentration of molybdenum was in both cases very similar (figure 1). The optimal molybdenum concentration was very wide, 0.01-10 p.p.m. (0.00001-0.001 %), the maximum possibly at 1 p.p.m.

In experiments with *Clostridium pasteurianum* the molybdenum content of the basal nutrient solution was 0.002 %. When $N_2$ and nitrate formed the sources of nitrogen the effect of molybdenum was much the same as with *A. vinelandii* (figure 2). When grown with ammonium nitrogen the growth of *Cl. butyricum* did not increase in any molybdenum concentration used (highest concentration 0.5 %).

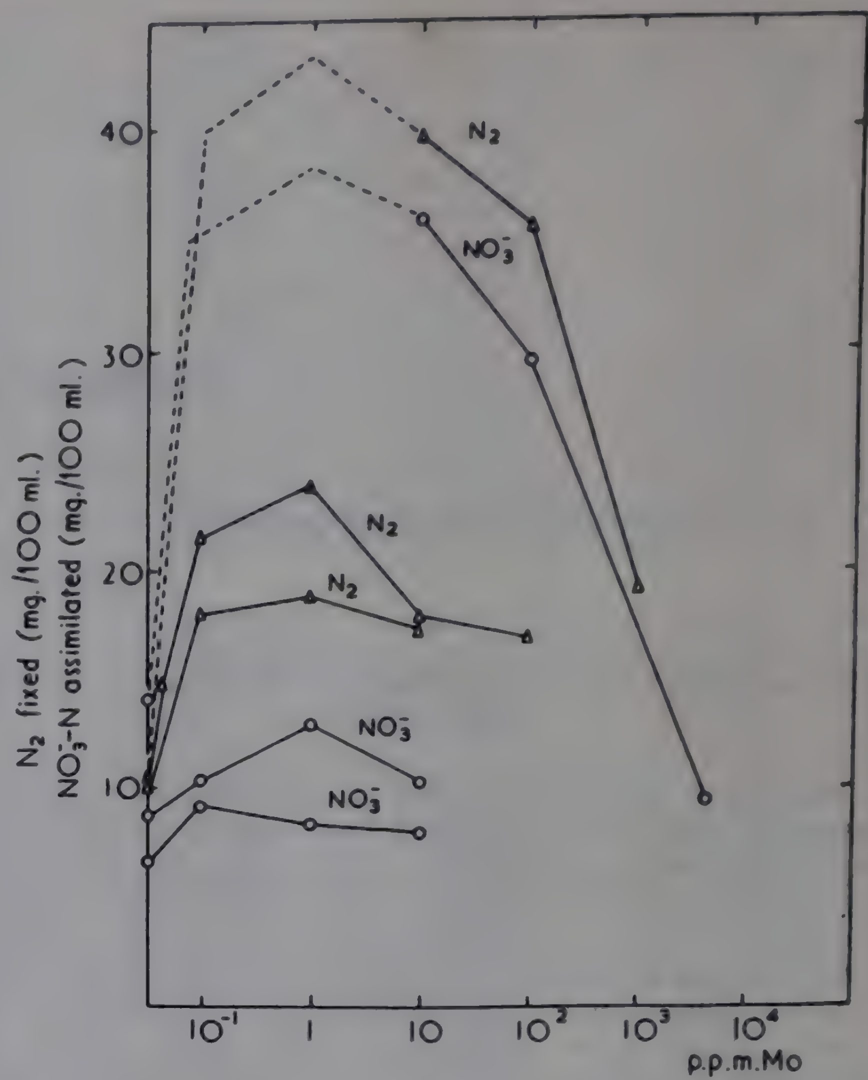



FIG. 1. — Dependence of $N_2$-fixation and $NO_3^-$-assimilation on the molybdenum content of the nutrient solution by *Azotobacter vinelandii*.

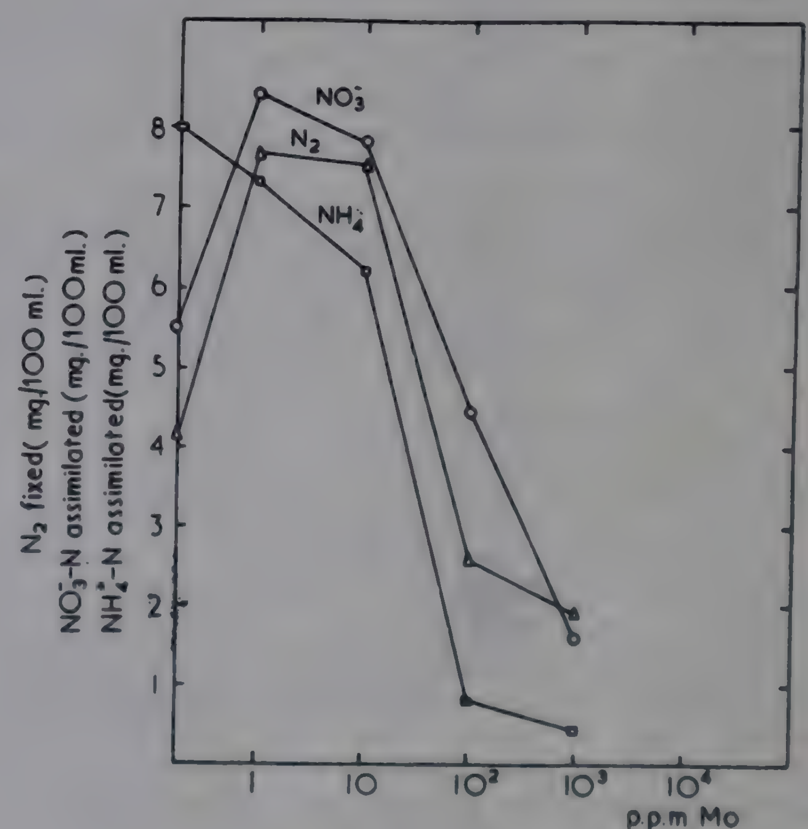



FIG. 2. — Dependence of $N_2$-fixation and $NO_3^-$-and $NH_4^+$-assimilation on the molybdenum content of the nutrient solution by *Clostridium butyricum*.

When ammonium nitrogen serves as the source of nitrogen, the effect of molybdenum on *A. vinelandii* in 0.001 % concentration was weak, but was intensified when the concentration increased, and reached maximum only in 0.1 % of the molybdenum concentration, *i.e.* in a concentration where the growth of *A. vinelandii* both with $N_2$ and nitrate is already relatively weak. The effect of molybdenum concerns therefore possibly quite different reactions when ammonium nitrogen serves as the source of nitrogen as when $N_2$ or nitrate form nitrogen nutrition. There is, however, uncertainty about the influence of molybdenum on the assimilation of ammonium nitrogen because the uptake of molybdenum from ammonium sulphate nutrient solution, the acidity of which tends to rise, may be poorer than *e.g.* from nitrate nutrient solution, and we did not estimate the molybdenum content of the bacteria.

The influence of vanadium on the growth of *A. vinelandii* with $N_2$ was about 70 % of the influence of molybdenum, and with nitrate about 90 %. The optimal concentration of vanadium was about 0.001 %. The optimum was wide with nitrate nitrogen, with $N_2$ narrower (figure 3). On the basis of the effect of vanad-

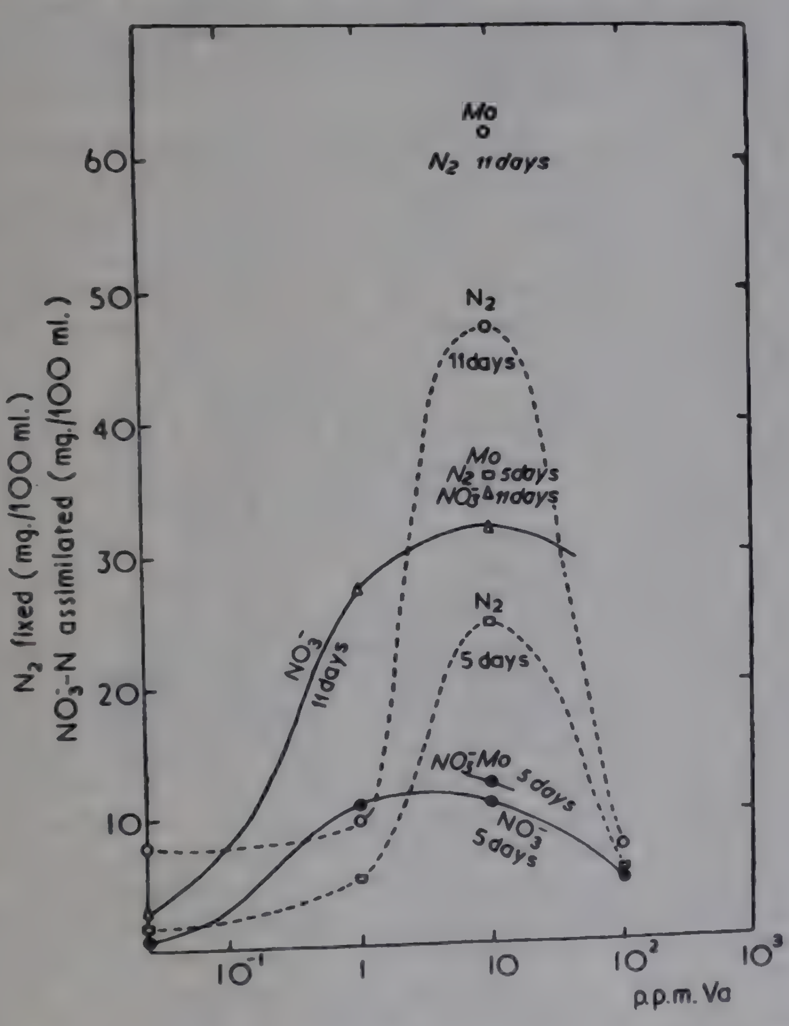



FIG. 3. — Dependence of $N_2$-fixation and $\overline{NO_3}$-assimilation on the vanadium content of the nutrient solution by *Azotobacter vinelandii*. For comparison the effect of molybdenum was estimated at 10 p. p. m.

ium it seems possible that vanadium replaces molybdenum even if the activity of the enzyme is somewhat lower.

The results obtained do not give any proof of the specific influence of molybdenum (or vanadium) in $N_2$-fixation, and of the different function of it in nitrate assimilation. They do not, however, refute this concept either As the concentration curves with molybdenum (and vanadium) are noticeably similar both in $N_2$ and $NO_3$-N experiments molybdo proteins could be expected to act as well in $N_2$-fixation as in the utilization of $NO_3$-N. Several enzymes have recently been found to be molybdo-flavoproteins (nitrate reductase, xanthin oxidase, liver aldehyde oxidase, hydrogenase). The nature of the nitrate reducing enzyme of *A. vinelandii* is unknown, but it may be a molybdo-flavoprotein. If the $N_2$-fixing enzyme should also be a molybdo-flavoprotein a noticeable similarity in the dependence of nitrogen fixing organisms on molybdenum, both with $N_2$ and nitrate nutrition, were in no way astonishing, even if the action of the enzymes concerns different reactions : activation of $N_2$ and reduction of $NO_3^-$.

The significance of iron in $N_2$-fixation is difficult to demonstrate because iron belongs to many of the key enzymes in the metabolism, and the requirement of it thus is remarkable whatever nitrogen nutrition the organism receives. Iron may, however, belong to the enzyme system acting in nitrogen fixation (*cf.* below). The observation of Richert and Westerfield (5) that both iron and molybdenum belong to the xantinoxydase is of special interest in this connection. If this observation is confirmed the enzyme activating $N_2$ may also contain both metals. The inhibitory effect of carbon monoxide on $N_2$-fixation, first observed by Wilson *et al.* (6), could be understood as an inactivation of iron or molybdenum, wherefore either, or both, metals could belong to the enzyme activating $N_2$. The inhibition which is specific for $N_2$-fixation and non-competitive, has been established both with *A. vinelandii*, blue-green algae, and leguminous root nodules. In our laboratory (7) it was recently observed that anaerobic $N_2$-fixation by *Cl. butyricum* is also inhibited by approximately the same concentrations of carbon monoxide as $N_2$-fixation with *Azotobacter*. The carbon monoxide concentration required for complete inhibition is for different organisms the following :
*A. vinelandii*, *ca.* 0.5 %; *Cl. butyricum*, 0.5-1.0 %; leguminous root nodules, *ca.* 0.05 %.

The greater sensitiveness against carbon monoxide of $N_2$-fixation in the root nodules of legumes probably depends on the leghemoglobin in the nodules (*cf.* below) which thus should become inactivated by lower concentration of carbon monoxide than the $N_2$-fixing molybdenum containing enzyme. On the basis of the inhibitory effect of carbon monoxide on $N_2$-fixation we have reason to presume that $N_2$ would combine with the heavy metal (iron or molybdenum) of the $N_2$-fixing enzyme, and thus become activated,

$$N_2 \rightarrow 2\,N\ (\rightarrow 2\,N^+ + 2\,e^-)$$

so that the activated oxygen or hydroxyl or hydrogen could combine with it in analogy with the activation of $H_2$ by hydrogenase. The requirement of energy is, however, much greater in the dissociation of $N_2$ than in that of $H_2$,

$$H_2 \rightarrow 2\,H\ 4.5\ \text{ev};\quad N_2 \rightarrow 2\,N\ 9.8\ \text{ev}.$$

Another possibility for the activation of $N_2$ is its being activated without dissociation of the molecule into atoms.

The very interesting observations made by Wilson and his associates (8) about the similarity, or almost identity, between hydrogenase and 'nitrogenase' deserve special mentioning in this connection. Since organisms with an obvious hydrogenase effect, but no $N_2$-fixing capacity, are found, the enzymes are not apparently identical. $N_2$ acts as an inhibitor of hydrogenase neither in *A. vinelandii* nor *Cl. butyricum*, but possibly in some photosynthetic bacteria.

Among other inhibitors of $N_2$-fixation $H_2$ (9) and $N_2$ (10) ought to be mentioned. The competitively inhibitory effect of hydrogen gas on nitrogen fixation both by *A. vinelandii*, blue-green algae, and leguminous root nodules has been observed. In anaerobic nitrogen fixation a corresponding inhibition has not been proved. The inhibitory effect of $H_2$ has been supposed to be due to its competition with $N_2$ for the enzyme.

Wilson and his associates found that $N_2O$ also inhibits $N_2$-fixation with *Azotobacter*, but not the assimilation of combined nitrogen ($NH_4$-N). In this laboratory (11) we have found that $N_2O$ inhibits the uptake of $NO_3$-N as well. In this case, however, the peculiar fact has been noted that if the experiment time is so short that, on the average, all cells have not had time to divide, the inhibitory effect of $N_2O$ is not observed (11a). If the experimental time is prolonged so that the multiplication of cells is well on way (the number of cells having at least doubled), $N_2O$ inhibits appreciably the assimilation of $NO_3$-N and correspondingly the growth of bacteria. In the case of *Cl. butyricum* the assimilation of nitrate is also inhibited by $N_2O$. It is doubtful whether any evidence as to the mechanism of $N_2$-fixation can be obtained from the inhibition by $N_2O$ in growth experiments. There is great similarity in structure, shape, and size between $N\equiv N = O$ and $N\equiv N$, and this may cause the observed inhibition (11, 31).

On the whole I think that too definite conclusions are often drawn from inhibition experiments. This is the case especially when gases are in question. When investigating $N_2$-fixation in water solution in the ultrasonic field Ellfolk and I (12) observed that carbon monoxide and $H_2$ inhibit $N_2$-fixation strongly. The influence of carbon monoxide is extremely effective. $N_2O$ also inhibits fixation completely in all concentrations investigated. In the latter case, when the gas is relatively soluble, the filling of the cavities with gas may be in question. Regarding $H_2$ and carbon monoxide this is not probable because argon does not inhibit $N_2$-fixation. As we understand it, carbon monoxide and $H_2$ are oxidized more easily than $N_2$ and compete thus with $N_2$ in the ultrasonic field, even if no heavy metal catalyst is in question.

Even as early as towards the end of last century nitrogen fixation was mostly supposed to occur directly *via* reduction to ammonia. On the basis of his oxidation-reduction theory Wieland presented the following mechanism for the reduction :

$$N\equiv N \xrightarrow{H_2} HN = NH \xrightarrow{H_2} H_2N\text{-}NH_2 \xrightarrow{H_2} 2\ NH_3$$

The reduction of $N_2$ to ammonia seemed to be a satisfactory explanation until Blom (13) reported that he had found hydroxylamine in *Azotobacter* cultures when $N_2$ served as the source of nitrogen. On the basis of his observation he presented the following pathway for $N_2$-fixation :

$$N\equiv N \xrightarrow{H_2O} HOHN\text{-}NHOH \xrightarrow{H_2} NH_2OH$$

Later Endres (14)showed that 'bound hydroxylamine' is formed in *Azotobacter* cultures both when $N_2$ and nitrate serve as nitrogen nutrition. In our laboratory 'bound hydroxylamine' was found also in the 'sterile' substrate of inoculated legumes. Since the hydration of $N_2$ seemed improbable I (15) advanced in 1940 the hypothesis that the oxidation of $N_2$ may be the best explanation for the formation of hydroxylamine in $N_2$-fixation. The heat of formation of hydroxylamine is in this case positive, 24.3 kcal. ($\frac{1}{2}\ N_2 + \frac{1}{2}\ O_2 + {}^3/_2\ H_2 \rightarrow NH_2OH$ aq). Both the oxidative and the reductive way also appear in Burris and Wilson's scheme (16) from 1945, representing the possible pathways for nitrogen fixation.

As far as I can see only the formation of 'bound hydroxylamine' actually speaks for oxidative$N_2$-fixation. If this can be explained as caused by secondary oxidation, *e.g.* of ammonia or amino acids, there is no proof against reductive $N_2$-fixation. We have therefore undertaken to investigate the chemical nature of 'bound hydroxylamine' as well as the mode of formation of hydroxylamine more closely.

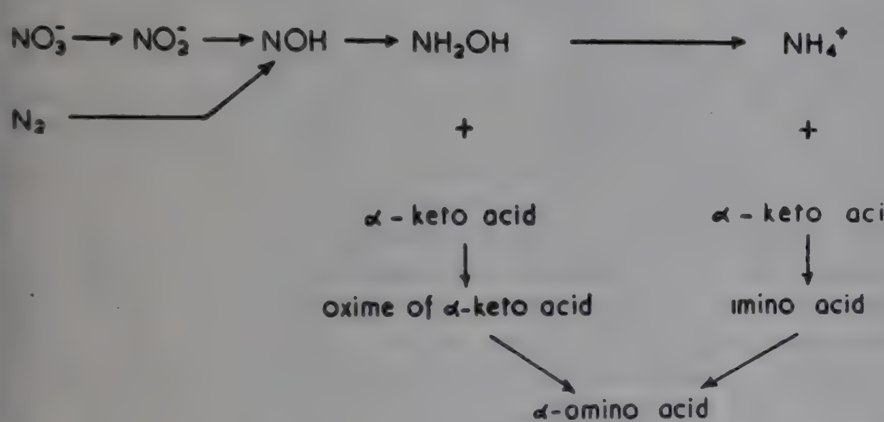
With 'bound hydroxylamine' the nitrogen fraction is meant which after acid hydrolysis on the oxidation with iodine gives nitrite. Endres presumed without proofs that oximes were in question. From the substrate of inoculated pea plants Laine and I (17) once succeeded in isolating a nitrogen fraction which contained 'bound hydroxylamine', and from which aspartic acid was formed on reduction. In accordance with this the substance must have contained at least a small amount of the oxime of oxalacetic acid. It has not, however, been possible to renew this result which has been the only proof in favour of oxime nitrogen. When some years ago enzymes which form hydroxamic acids from hydroxylamine, and carboxylic acids or their amides were found in animal organisms (18), micro-organisms (19), and green plants (20) it became apparent that 'bound hydroxylamine' may contain also these compounds, or be composed of them alone. In this connection a very interesting result by Nord *et al.* (21) has to be mentioned. They found that nitrate in cultures of *Fusarium* is reduced only to hydroxylamine, and that pyruvic acid acts as an acceptor of hydroxylamine.

Saris and I have investigated the formation of 'bound hydroxylamine' in nitrate and nitrite assimilation and in $N_2$-fixation. As test organisms we have used *Torulopsis utilis* and different strains of *Azotobacter*. The investigation has met with great methodic difficulties. For the present our results concern only *Torulopsis utilis*. After we had developed the method first to exclude the amino acids by ion exchange resin (Amberlite IR-120), then to reduce the 'bound hydroxylamine' to an amino group, and to determine by paper chromatography the amino compounds formed we were able to show that oximes of pyruvic acid, $\alpha$-ketoglutaric acid, oxalacetic acid, and glyoxylic acid are formed with nitrite as source of nitrogen (22). Most of the 'bound hydroxyl-

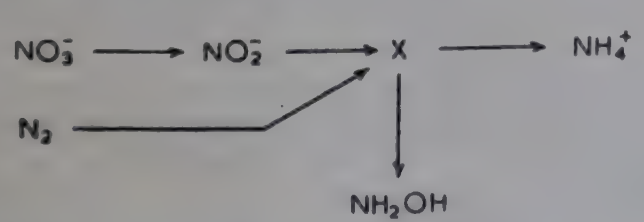
amine nitrogen' was oxime nitrogen (80-90 %). The rest remained in the resin together with amino acids. We have not as yet been able to ascertain the nature of this extremely small nitrogen fraction.

The oximes of α-keto acids are formed from hydroxylamine and keto acids without any enzymes. Some years ago I determined with Alfthan (23) the reaction rate between hydroxylamine and keto acids, and found it so great with the key α-keto acids that 'free hydroxylamine' can hardly appear in cells under normal conditions.

Hydroxylamine is apparently formed as a reduction product of nitrite. This is shown by the rapid formation of 'bound hydroxylamine', and the afterwards occurring decrease of it in *Torulopsis utilis*, which I earlier observed in nitrate solution with Csaky (24). Our present investigations with Saris have given similar results by using nitrite. Accordingly, the oximes of α-keto acids which we found have not been formed from amino acids *via* secondary oxidation. These results thus give some support to my old hypothesis that there are two different pathways of formation of amino acids when nitrate or $N_2$ serve as the source of nitrogen. Unfortunately, we have not yet been able to confirm the formation of the oximes of α-ketoacids in *Azotobacter* growing with $N_2$ and the nature of the 'bound hydroxylamine' formed in $N_2$-fixation is therefore still unknown.

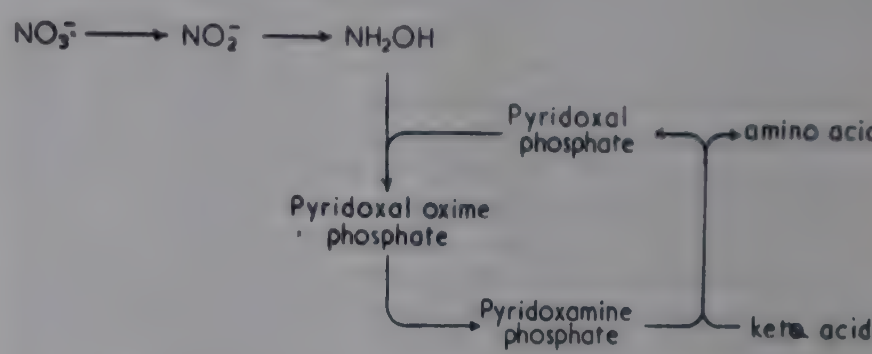



The possibility that hydroxylamine is not formed as an intermediate, but as a by-product, for instance in the following way :




is also to be taken into consideration. There are, however, no proofs in favour of this view.

At the moment the reduction of the oximes of keto acids to the corresponding amino acids is still obscure even if observations about this event (*e.g.* 'oximase' by Yamafuji, 25) begin to appear in the literature. If it turns out that nitrate using organisms generally do not reduce oximes of keto acids it is probable that the reduction takes place at the hydroxylamine stage, and that the oximes found are only by-products. Silver and McElroy (26) quite recently published very interesting results concerning the reduction of nitrate and nitrite with the mutants of *Neurospora*. As pyridoxin seems to be connected with the reduction of nitrite in some way, these authors advanced the hypothesis that hydroxylamine which arises as the reduction product of nitrite forms oxime with pyridoxal phosphate. This hypothetic oxime should be reduced to pyridoxamine phosphate, which transaminates with keto acids, the corresponding amino acids being formed :




Our findings show that hydroxylamine reacts directly with α-keto acids. It may, however, also react with pyridoxal phosphate. There is at the moment no proof for Silver's and McElroy's very interesting hypothesis.

It is very probable that also with nitrate and $N_2$ as nitrogen nutrition the amino acid synthesis from ammonia is the main pathway in a quantitative sense. This concept is supported by the fact that $^{15}N$ is mostly enriched into glutamic acid (8), the enzymatic synthesis (from ammonia and α-ketoglutaric acid) of which is a common reaction in organisms. A smaller, or even considerable, part of nitrogen can, however, be transformed over oximino acids to amino nitrogen. The enrichment of $^{15}N$ foremostly into glutamic acid is even in this case natural.

The results about the formation of hydroxylamine (Egami's observations, (27) about 3 different reductases, nitrate-, nitrite-, and hydroxylamine-reductase, in the reduction of nitrate to ammonia are extremely important) and its reaction with α-keto acids in cells give a certain even if not very clear idea of the position of hydroxylamine in nitrate reduction. The situation regarding its formation in $N_2$-fixation is more vague. Endres observed, as mentioned before, that 'bound hydroxylamine' is formed in *Azotobacter* cultures having $N_2$ as the sole source of nitrogen. According to him no 'bound hydroxylamine' was formed with ammonium nitrogen. In the 1940's Järvinen and I (28) investigated the formation of 'bound hydroxylamine' with different sources of nitrogen more closely. Using a suspension of *A. vinelandii* the formation of 'bound hydroxylamine' could be observed as soon as after 1-2 hours both with nitrate and $N_2$. By that time no increase in 'bound hydroxylamine' was perceptible in suspensions containing ammonium nitrogen. After 3 hours 'bound hydroxylamine' began to appear even in this suspension, which means that the amino group or ammonia can also be oxidized. This of course weakens the argument of the formation of hydroxylamine as an intermediate in aerobic $N_2$-fixation which the formation of 'bound hydroxylamine' with $N_2$ or nitrate as the sole source of

nitrogen would mean. In growing aerated cultures of *A. vinelandii* with urea or $(NH_4)_2SO_4$ as nitrogen source, no 'bound hydroxylamine' could however be found in our recent investigations.

The main question for the knowledge of the chemical mechanism of $N_2$-fixation is at the moment in my opinion : is the reaction oxidative or reductive ? Any definite answer to this question cannot yet be given. If it can be proved experimentally that 'bound hydroxylamine' is formed as an intermediate in $N_2$-fixation before ammonia, I should regard the oxidative mechanism of $N_2$-fixation proved. If, again, hydroxylamine is formed secondarily from ammonia, there are in my opinion no proofs in favour of the oxidative mechanism, and the reductive mechanism is then a probable one. In this case the incongruity existing between the observations on anaerobic (*Clostridium*) and aerobic (*Azotobacter*) $N_2$-fixation should be settled. In our laboratory we have not been able to find even a trace of 'bound hydroxylamine' in anaerobic $N_2$-fixation (22). I think therefore that there are no proofs in favour of oxidative $N_2$-fixation and that on the basis of experimental results a reductive one may accordingly be most probable in anaerobic $N_2$-fixation. On the other hand the hypothesis of a different mechanism in aerobic and anaerobic $N_2$-fixation does not seem very attractive. The absence of 'bound hydroxylamine' in clostridia cultures can of course be explained even in the case that hydroxylamine is formed as an intermediate. Jensen (29) has recently suggested that the NOH group formed as an intermediate in anaerobic $N_2$-fixation by *Clostridium* may be reduced so rapidly under the conditions existing (very low redox potential, liberation of hydrogen) that hydroxylamine would be absent from the clostridia cultures.

If $N_2$-fixation occurs *via* oxidation, the question arises which is the first oxygen containing compound. As possible compounds $N_2O$, NO, $(NOH)_2$, the radical NOH, and nitramide have been proposed. The first of these has attracted attention as an eventual intermediate because it inhibits $N_2$-fixation (*cf.* above). Since $N_2O$ is not utilized by $N_2$-fixing bacteria it has generally been disregarded as an intermediate. Quite recently Mozen and Burris (30), however, observed in their experiments with $^{15}N$ labelled $N_2O$ that azotobacter and soybean nodules utilize it in very dilute solutions. Roberts (31) thinks, however, that the positive results may depend on the formation of $N_2$ from $N_2O$ which is not in accordance with the findings of Mozen and Burris. Be that as it may, the non-utilization of $N_2O$, no more than any other possible intermediate, cannot be regarded as a conclusive proof against it as an intermediate. There is on the other side no proof in favour of $N_2O$ in $N_2$-fixation.

The formation of NO is not supported by any proofs. I have, however, introduced it as a possible intermediate because its formation as the primary product in $N_2$-fixation by ultrasonic waves is probable. It could join the reduction path of nitrate.

Hyponitric acid has often been discussed as an intermediate in nitrate reduction and in analogy also in $N_2$-fixation. Egami (27), however, could not reduce hyponitric acid with a bacterial preparation which reduced nitrite to ammonia. Accordingly, there is a proof against the assumption that hyponitric acid acts as an intermediate in nitrate reduction.

Both Roberts (31) and I (32) have introduced the radical NOH as a possible intermediate before hydroxylamine. Allen and van Niel (33) have introduced nitramide as a probable intermediate in denitrification. The question is still unclear. There is no proof in favour of its possible intermediary nature in $N_2$-fixation.

### *Leghemoglobin*

The red pigment formed in leguminous root nodules, for which Kubo (34) first demonstrated a hemoglobin nature, is according to observations in our laboratory involved in $N_2$-fixation (35) in a surprising degree. Nodules formed by ineffective strains do not contain this pigment, and effective nodules loose their $N_2$-fixing ability when the pigment changes into green. (The porphin ring opens hereby through oxidation. The green pigment still contains iron and protein. In acetic acid they are split off and biliverdin is formed. The green pigment is thus an intermediate between leg-hemoglobin and bile pigments).

A clear chemical difference had thus been found for the first time between effective and ineffective nodules. It was natural that the red pigment of root nodules, which I called leghemoglobin, was subjected to intense investigation. In the experiments by Ellfolk and me (35) to purify and characterize leghemoglobin more closely we found that it separated electrophoretically into two components of which the faster component has an isoelectric point of 4.4 and the slower 4.6. The molecular weight of the former is about 17 000, and the iron content $\sim$ 0.35 %, indicating one hem group per mole. We consider this component to be pure leghemoglobin. It has the lowest isoelectric point of all the known hemoglobins and similar oxygen carriers. Its histidine content is very low, hardly 1/4 of that of myoglobin. In this respect, as well as in respect of the IP, the erythrocruorins are between leghemoglobin and myoglobin. The molecular weight of the slower component is noticeably greater, perhaps $\sim$ 45 000, also containing only one hem group. Its spectrum is qualitatively similar to that of the former component. We have therefore assumed that it contains leghemoglobin in some way combined with an iron-free protein. Further particulars about the structure of this component are lacking. The red pigment is formed in the root nodules in the cytoplasm of the root cells outside the bacteria or bacterioids as a result of symbiosis. The pigment is probably in the first place formed by the host plant. What part bacteria play in its formation is still unknown.

When comparing the leghemoglobin content of the nodules formed on the roots of the pea plants by effective strains of *Rhizobium* with the amounts of fixed nitrogen (total N of pea plant *minus* N of seed) we observed in 1943 a parallelism between them. In our later experiments (37) with many *Rhizobium* strains with different efficiency this parallelism also has been found (figure 4). Some years ago Jordan and Garrand (38) also confirmed this observation. We can roughly conclude that the

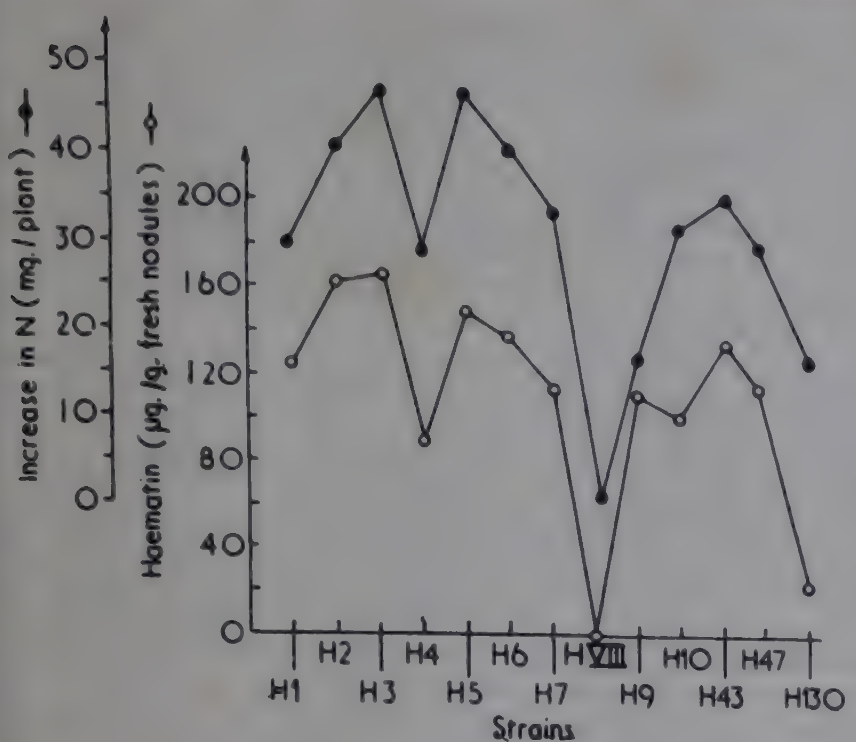



FIG. 4. — Hematin content of the nodules of 'Torsdag' pea inoculated with different bacterial strains. Period of growth : 23. IV-17. VI. 1947. Plants in full bloom at the end of the period, nodules chiefly red, a few green nodules in some experiments. The low absorption of inactive H VIII nodules is subtracted from each experiment.
●———● Increase in N, mg./plant.
○———○ Hematin, µg./g. fresh nodules.

effectivity of the nodules is the greater the higher the leghemoglobin content of the nodules.

The indispensability of leghemoglobin for $N_2$-fixation in root nodules is a remarkable fact. It of course arouses the question what function this pigment has in $N_2$-fixation. No satisfactory answer to this question has been obtained during the time of more than ten years which has passed since the indispensability of the pigment for $N_2$-fixation was observed. Since leghemoglobin, or some other closely related hemoglobin, has not been found in free living $N_2$-fixing organisms, nor in the root nodules of alder which fix $N_2$ liberally, the interpretation seemed more natural that leghemoglobin acts as a carrier and storer of oxygen and is therefore indispensable in the oxygen deficient conditions prevailing in leguminous root nodules. No experiments have, however, given results which support this view. In Keilin's laboratory Smith (39) could not find any influence of the red pigment on the oxygen uptake of detached root nodules. Carbon monoxide also had no effect on the oxygen uptake of nodulated roots when these were attached to the plant. In our laboratory (40) no correlation between the leghemoglobin content of the nodules and the oxygen uptake by them could be found. In these experiments nodules with very different leghemoglobin content were used (table I).

I have also discussed the possibility that leghemoglobin may reduce hydroxylamine if it is formed in the nodules. According to Colter and Quastel (4), hemoglobin of blood, and on the basis of the results in our laboratory (42) also leghemoglobin, reduces hydroxylamine quantitatively to ammonia in the presence of ascorbic acid or cysteine. Leghemoglobin seems to be the main, or possibly only, factor which causes the reduction of free hydroxylamine in root nodules.

A question closely connected with the effectivity of different *Rhizobium* strains is the decomposition of leghemoglobin to green pigment in the nodules. In pea plants inoculated with less effective *Rhizobium* strains it can generally be observed that the nodules begin to turn green even under favourable growth conditions before the flowering stage, while very effective nodules under the same conditions remain red weeks after the start of flowering and turn green only when the vegetative growth of the plant is coming to an end. Under unfavourable light conditions even very effective nodules may turn green earlier. Since the retention of leghemoglobin in the nodules as long as possible is of decisive importance for the fixation of $N_2$, and thus for the growth of leguminous plants, the decomposition of leghemoglobin to green pigment is not only of theoretical but also of great practical importance. Hydrogen peroxide causes *in vitro* the opening of the porphin ring through oxidation and forms a green pigment from hemoglobin in the presence of ascorbic acid. Hydrogen peroxide may also be the oxidizing agent in the root nodules, and thus cause the decomposition of leghemoglobin to green pigment. According to our determinations there is much more of ascorbic acid in the root nodules of pea plants than in the roots (43). The catalase content of the red nodules is many times higher

TABLE I

*$O^2$-uptake by excised root nodules of pea*

| *Rhizobium* strain | Age of pea plants (days) | Hematin content of nodules (mg./g. dry substance) | Hematin in leghemoglobin | R.Q. | $Qo_2$ | Relative value | $\frac{Qo_2}{\text{Hematin}}$ | Relative value |
|---|---|---|---|---|---|---|---|---|
| H6 effective | 49 | 0.735 | 0.560 | 1.00 | 2.88 | 202 | 3.9 | 186 |
| H10 effective | 43 | 0.559 | 0.384 | 1.05 | 3.94 | 275 | 7.5 | 357 |
| HVIII ineffective | 45 | 0.175 | 0.000 | 1.10 | 4.30 | 300 | 24.5 | 1167 |
| H130 slight effective | 48 | 0.253 | 0.078 | 1.00 | 3.81 | 266 | 15.1 | 719 |
| H43 effective | 43 | 0.853 | 0.678 | 1.00 | 3.90 | 272 | 4.5 | 214 |
| H47 effective | 50 | 0.700 | 0.525 | 0.92 | 1.43 | 100 | 2.1 | 100 |
| H301 effective | 46 | 1.014 | 0.839 | 0.98 | 3.73 | 262 | 3.8 | 181 |
| H313 effective | 45 | 0.712 | 0.537 | 1.04 | 3.34 | 234 | 4.7 | 224 |

than of the green ones (*Katalasefähigkeit* of red nodules of pea approx. 3.0, of green nodules approx. 0.6) (44). Whether this decrease of catalase activity leads to an increase in hydrogen peroxide of the nodules is still unknown because of difficulties in estimating the hydrogen peroxide content of the nodules.

The transformation of the effective nodules of legumes into green when growing plants are subjected to complete darkness also belongs to this category of problems (35). The red pigment of pea nodules turns into green within a period of 2-3 days, while, for instance, the decomposition of leghemoglobin in soya nodules is very slow and takes a couple of weeks. The reaction is even in so far different that in the pea nodule the globin combined with the hem group is retained undenaturated when the porphin ring opens, while in soya nodules the globin becomes denaturated so that biliverdin cannot any longer be formed with acetic acid from the green pigment (45).

### *$N_2$-fixation with excised root nodules*

Investigation of symbiotic $N_2$-fixation is difficult and slow because both the host plant and the root nodules belong to the system. For a long time the concept predominated that excised root nodules do not fix nitrogen. In our laboratory we were able to show in 1937 what we take to be a definite $N_2$-fixation also with excised pea nodules (46). Using large amounts of nodules, 10-15 g. for each sample, we could obtain relatively homogenous nodule masses for each experiment so that the amount of nitrogen at the beginning and end of the experiment could be determined with such an accuracy that a fixation of 3 % of nitrogen could be established. In our experiments the addition of oxalacetic acid promoted nitrogen fixation (perhaps by stabilizing leghemoglobin). In many experiments an increase of 3-10 mg. (4-12 % of tot. N) in nitrogen was found. The significance of these results was discussed until the nitrogen fixing capacity of excised root nodules by and by became confirmed in the experiments made in Wisconsin with $^{15}N$. The only difference between our earlier results and the ones obtained in Wisconsin is that addition of oxalacetate was not found in Wisconsin to promote $N_2$-fixation, but rather to lessen it. When Dr. Burris last summer worked in our laboratory he performed an experiment with excised root nodules of pea using $^{15}N$ and found that addition of oxalacetate activated $N_2$-fixation considerably (about 30 %) (5 flasks with oxalacetate, 5 without). It seems thus that the composition of root nodules grown under our light conditions is somewhat different from that of those grown in Wisconsin. It is probable that by using excised root nodules many important questions concerning symbiotic $N_2$-fixation can be studied more succesfully than with growing leguminous plants. It may be mentioned that last summer in our laboratory Burris *et al.* found a clear, in some experiments even a strong $N_2$-fixation even with excised root nodules of alder (47).

### *Determination of $N_2$-fixation in legume cultures in soil*

In this connection there is no possibility to take up problems concerning the varying activity of different *Rhizobium* strains, nor the specificity of rhizobia, particularly as the phenomenon has not yet been elucidated. I would like to mention, however, a method to determine how much combined nitrogen legumes (pea) take up from the soil under field conditions in different types of soil and how much $N_2$ it gets from the air *via* root nodules. This method has been developed in our laboratory and is founded on the observation that a young pea plant inoculated with completely ineffective pea rhizobium (H VIII in our laboratory) after the formation of the first nodules is in some way immune against other strains of pea rhizobia for about six weeks. A pea plant infected with the H VIII strain can thus grow only on combined nitrogen, while the pea plant inoculated with an effective strain gets its nitrogen nutrition *via* its root nodules from the air ($N_2$). They can of course at the same time take combined nitrogen with their roots. Briefly, the experiment is performed in the following way : In the plant department of our laboratory *e.g.* 2 000 pea plants are grown in water culture without combined nitrogen. A thousand of these plants are infected with the ineffective H VIII strain and 1000 with a strain of *Rhizobium* as effective as possible. The cultures are arranged so that external rhizobium infection is avoided as far as possible. When nodules have appeared on the roots of most plants they are planted in the experimental plots. It is astonishing to see how little the pea plant infected with the ineffective strain grows even in good soil where oats or wheat give good yields without nitrogen fertilization. As H VIII peas can get nitrogen nutrition only from the soil, peas inoculated with an effective strain also from the air, the difference in the amount of nitrogen between both pea groups derives from $N_2$ fixed by their root nodules. If effective nodules begin to appear on the roots of H VIII peas, which in our experiments has happened in one month at the earliest, in some soils only in six weeks, after the planting, the experiment has to be stopped at once. In experiments of this kind we have observed that the pea takes very little combined nitrogen from the soils where no nitrogen fertilizers have been used before planting. In two field experiments in light clay soils where *e.g.* oats grew well the pea inoculated with a very effective *Rhizobium* strain obtained 80-90 % of its nitrogen from the air, in pot experiments with different types of soils 60-90 % (48, 49).

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# The relation between solvent drag and active transport of ions

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(*Received* 21 *April* 1955)

### *Definition of active transport*

The term active transport is generally used to designate a transport which cannot be accounted for on the basis of the physico-chemical properties of the two phases separated by a living membrane. This definition implies that the active transport must be ascribed to chemical or physical processes taking place in (or at) the membrane phase.

According to this definition transfer taking place due to difference in concentration or in activity coefficient for the substance in question is not active transport; neither is a transfer of an ion due to an electric potential difference (p. d.) to be considered as active transport.

### *The solvent drag*

There is, however, one additional force of physical nature which may influence the movements of dissolved substances, namely solvent drag, *i.e.* the force exerted upon the solute by the flow of solvent through the membrane. In contrast to the forces arising from differences in concentration, activity coefficient and electric potential, the drag force cannot be deduced solely from the physico-chemical properties of the bathing solutions. It can easily be shown that it is a function of the membrane structure. Taking, for instance, the fluid circuit hypothesis of Ingraham, Peters and Visscher (8) the salt transport across the intestinal mucosa might arise as the resultant of a flow of salt solution from the lumen side to the blood side through a set of relatively large pores, and a pumping back of pure or nearly pure solvent through a set of narrower pores. Such a process would not give rise to characteristic anomalis in the properties of the body solutions.

### *Estimation of the solvent drag*

In case we want to restrict the use of the term 'active transport' to cases where the underlying mechanism consists in one or more chemical reactions, it would be desirable to devise means to measure the drag force. It is, as a matter of fact, possible to obtain an estimate of the solvent drag on a given ion species from the behaviour of suitable test substances. Test molecules diffusing in the direction of solvent flow will be speeded up and those going 'upstream' will be slowed down. The rates in the two directions can be measured simultaneously by aid of two batches of the test substance, labelled differently with isotopes.

The logarithm to the flux ratio influx/outflux) is a direct measure of the drag force on the test substance if the latter is uncharged and if furthermore it is added to give the same activity on both sides of the membrane.

The line of reasoning behind this statement is the following : influx and outflux (amount passing in unit time through unit area) may be considered as measures of the rate constants for the forward and backward reactions of the permeation process. As it is well known, we have for a chemical process that the equilibrium constant, $K$, is equal to the ratio between the rate constants $k_1$ and $k_2$. If the influx and the outflux of the test substance are designated $M_{in}$ and $M_{out}$ we then have :

$$-\Delta F = RT\ln K = RT\ln (M_{in}/M_{out}) \qquad (1)$$

In other words, the free energy change, which has the dimension of a potential difference, is determined by the flux ratio. In case the test substance is present at the same concentration (or strictly speaking, activity) on both sides, the difference in free energy shown by the test substance must be solely due to the solvent drag. It is evident that solvent drag will arise only when the solute moves in a continuous water phase. In other worlds : the membrane must be a pore membrane. If water as well as solute molecules penetrate by dissolving in a homogeneous lipoid film, there can be no solvent drag.

Now it can be shown (Koefoed-Johnsen and Ussing, 10) that the drag force arising in a pore membrane is determined by the equation :

$$\ln (M_{in}/M_{out}) = \frac{\Delta_w}{D}\int_0^{Xo} \frac{1}{A}\, dx \qquad (2)$$

where $\Delta w$ is the net transfer rate of water per unit area, $D$ is the diffusion coefficient of the test substance, $A$ is the fraction of the unit area which is available for diffusion, $x_0$ is the thickness of the membrane and $x$ is the distance in the membrane phase from one of the boundaries. This equation is only correct if (a) the test

substance is strongly hydrophilic so that it will follow the pores without dissolving in the membrane material and (b) the pores are all of the same shape; on the other hand they are allowed to have any conceivable form.

It will be noticed that $D$, the free diffusion coefficient, is the only quantity in the right hand expression which depends on the individuality of the test substance.

$$\Delta_w \int_0^{X_0} \frac{1}{A}\, dx$$ is common to all hydrophilic molecules and can be calculated from the flux ratio of a test substance whose diffusion coefficient is known.

If the pores belong to two or several size groups, the membrane may be considered as a mosaic; for each size group an equation of the above form is valid.

Returning to the fluid circuit hypothesis, we recall that two sets of pores are required, one having large pores which allow salt to follow the water and another one having narrow pores suitable for returning more or less pure solvent. $\Delta w$ is taken to be positive for the large pores, yielding a flux ratio larger than one. For the small pores, where $\Delta w$ is negative, we should of course get a flux ratio smaller that one, but if the test molecules are chosen sufficiently large, none of them will pass through the small pores. Consequently the flux ratio found will be a measure of the maximum drag force available through the operation of the whole 'fluid circuit pump'.

### *Active transport of Na*

To make the discussion more specific we may concentrate on the problem whether or not water drag plays any significant part in the transport of Na across living membranes. Na is chosen because in recent years a large amount of evidence has been forthcoming, indicating that many, if not all, animal cells handle Na in a way which cannot be explained on the basis of existing concentration differences and electric potential differences (Krogh, 11; Ussing, 17, 18; Hodgkin, 6). In some cases the active nature of the Na transport is surmised on the basis of evidence which is rather incomplete. Thus when most authors agree that the low intracellular Na concentration in nerve and muscle fibres is due to active extrusion of Na, it is based on the assumption that the activity coefficients for Na inside and outside the fibres do not differ appreciably. In the case of muscle Ling (12) has, as a matter of fact, advocated the alternative hypothesis, namely that the Na is kept out of muscle by lack of space for the large Na ions in the lattice of muscle protein molecules. Incidentally, the fact that Li-ions which are even larger than Na-ions can penetrate into muscle fibres to give a concentration which is approximately equal to that outside seems hard to explain on the basis of Lings hypothesis. In the case of most other cell types the problem is even more difficult because we know neither the potential difference nor the correct activity coefficient to be applied for internal Na.

If we pass from cell membrane to multicellular membrane the situation is somewhat clearer in that the activity coefficient can be estimated with a fair degree of accuracy for both bathing solutions. In several cases, however, the measurement of the electric potential difference across the membrane under normal functioning conditions meets with difficulties (example : kidney tubules). There remains, nevertheless, a number of epithelial membranes which permit the determination of concentrations, activities and potential difference and which, further, can be kept alive in the isolated state, thus allowing a more detailled study of the penetration kinetics. Notably the skins of anurans (frogs and toads) have turned out to be well suited for the study of ionic permeability. It can be shown in different ways (Ussing, 15, 16; Ussing and Zerahn, 19), that these skins are able to perform a powerful active transport of Na from the outside to the inside medium and that, furthermore, this Na-transport is solely responsible for the electric potential difference which these organs maintain when in contact with identical salt solutions on both sides.

### *The short-circuited frog skin*

Probably the most instructive and unambiguous method for demonstrating this active transport mechanism is the short-circuiting technique (Ussing and Zerahn, 19). The principle is the following : (compare figure 1) The skin, $S$, is placed as a membrane

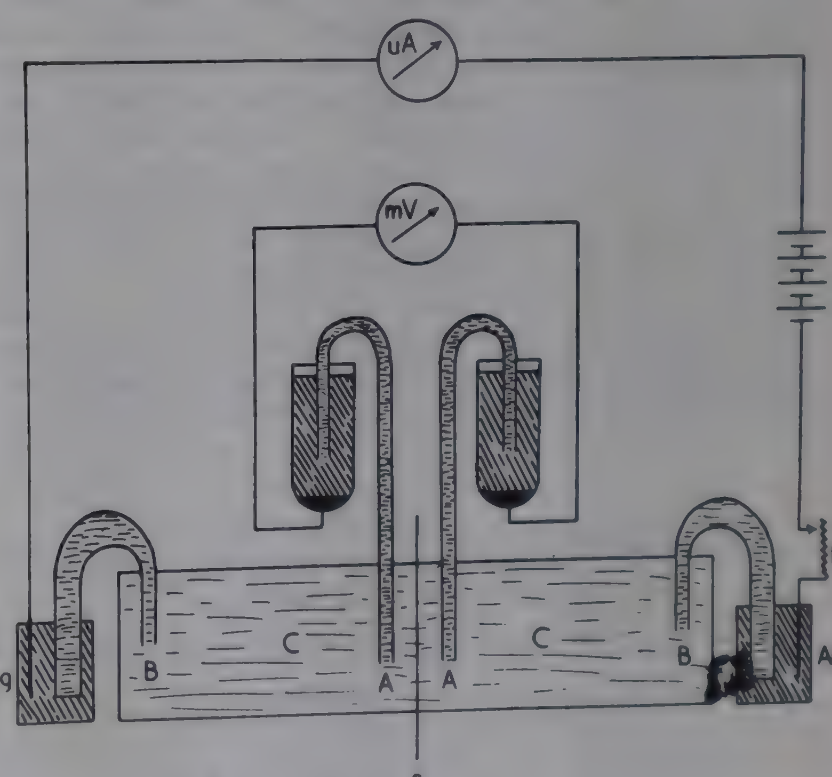


FIG. 1.

separating the inside and outside solutions (usually Ringer) which are kept well mixed and aerated by a pumping device not shown in the figure. The potential drop across the skin is measured between the two calomel electrodes $A$ and $A^1$ which are provided with Ringer-agar bridges opening on either side close to the skin. Another pair of electrodes $B$ and $B^1$ are connected through a micro-amperemeter, a variable resistance and

an electric battery. When the outer circuit is disconnected the skin maintains a potential difference between the inside and outside solution, which may be as high as 130 mV (the inside +). When the outer circuit is established, it is possible to adjust the current strength by aid of the variable resistance to such a value that the potential drop across the skin becomes zero. Now, if the terminals of a source of electric potential, for instance a battery, are connected in such a way that the potential difference between them becomes zero it is by definition short-circuited, and will give its maximum current. So also with the frog skin. The skin does not 'know' that the battery provides the electromotive force necessary to overcome the outer resistance, including that of the bridges and the solutions. All that the electromotive force of the skin has to do is to overcome its own internal resistance. The short-circuit current generated by the skin can be directly read on the microamperemeter.

It is now possible to compare the current output with the net transfer rates of all the ion species present in the bathing solutions, using radioactive tracers to measure outflux and influx. For the ion species present in Ringer, the results can be summarized as follows : Cl diffuses through the skin in both directions at the same rate. Consequently the net chloride transfer is nil. The same is the case for the K ion. Ca diffuses so slowly that its contribution if any to the current output is below the accuracy of measurement. The Na ions, however, behave differently. The influx of this ion is 20 to 100 times the outflux and the difference between these two values, the net flux, is exactly equal to the electric current output. This can most convincingly be demonstrated using the two tracers $^{24}$Na and $^{22}$Na for the simultaneous determination of influx and outflux (table I). It is seen that the net Na flux, $\Delta$Na, is identical within the limits of accuracy to the electric current output. Obviously this Na transfer represents active transport. The Na concentration is the same on both sides of the skin and the potential difference is maintained at zero during the experimental period.

TABLE I

*Short-circuit current and sodium flux values for a number of short-circuited frog skins* (Rana temporaria) (*Ringer solution on both sides*)

| | μamps/cm² | | | |
|---|---|---|---|---|
| | Na in | Na out | ΔNa | Current |
| I | 20.1 | 2.4 | 17.7 | 17.8 |
| II | 11.1 | 1.5 | 9.6 | 9.9 |
| III | 40.1 | 0.89 | 39.2 | 38.6 |
| IV | 62.5 | 2.2 | 60.3 | 56.8 |
| V | 47.9 | 2.5 | 45.4 | 44.3 |

*Active Na transport across other epithelial membranes*

Active Na transport of the type found in the amphibian skin is also at work in several other epithelial organs. Dr. Leaf (13), in our laboratory, has demonstrated that the short-circuit current output generated by the urinary bladder of the toad is again solely due to active Na transport.

Also the isolated large intestine of the toad maintains an electric potential difference which can be shown to be due to active Na transport (Andersen and Ussing, 1, in preparation). During short-circuiting this organ will give electric current for many hours and the flux analysis shows that all the current comes from active transport of Na (table II).

TABLE II

*Sodium flux values and short-circuit current for 3 isolated toad large intestines. Both sides Ringer. pH 8.3*

| | μamps/cm² | | | | p.d. (mV) before shorting |
|---|---|---|---|---|---|
| | Na in | Na out | ΔNa | current | |
| I | 47 | 21 | 26 | 31 | 37 |
| | 44 | 24 | 20 | 24 | |
| II | 34 | 9 | 25 | 23 | 16 |
| III | 37 | 16 | 21 | 20 | 30 |

Similar conditions are found in the caecum epithelium from the guinea-pig. When stripped off and placed as a membrane between Ringer solutions at body temperature, it develops a p.d. of 6 to 20 mV. During short-circuiting it generates a considerable current which again is due to active Na transport (compare table III). It

TABLE III

*Sodium flux values and short-circuit current for 6 isolated guinea-pig intestinal mucosa (caecum)*

| | μamps/cm² | | | | p.d. (mV) before shorting |
|---|---|---|---|---|---|
| | Na in | Na out | ΔNa | current | |
| I | 203 | 103 | 100 | 62 | 13 |
| II | 178 | 114 | 64 | 67 | 10.8 |
| III | 262 | 200 | 62 | 76 | 7.8 |
| IV | 234 | 174 | 60 | 54 | 9.7 |
| V | 245 | 137 | 108 | 75 | 8.6 |
| VI | 132 | 90 | 42 | 17 | 11.0 |

Influx (Na in) determined with $^{22}$Na, outflux (Na out) with $^{24}$Na.

Influx means flux from lumen to serosal side, outflux means flux from serosal to lumen side. Medium : both sides Ringer. pH : 7.8, except in experiment V where it was 7.1. In all cases was the inside (serosal side) positive relative to the outside.

will be seen, however, that the net Na transport is generally larger than the current output. This obviously means that some other active ion transport process brings about a transfer of charges which is opposed to that resulting from the Na transport. Whether this transfer of charge is due to the active transport of a positive ion in the direction opposite to that of Na, or it is due to a transport of a negative ion in the same direction has still to be found out.

### *Properties of Na transport mechanism; response to neurohypophyseal extracts*

The Na transport mechanisms of amphibian skin, urinary bladder and large intestine show many similarities. All three seem to be dependent on aerobic metabolism and all are inhibited by DNP. Time does not permit a detailled discussion of the effects of drugs and other metabolically active agents on these systems. There is, however, one reaction which is common to the three organs and which is of particular interest for the present inquiry : when neurohypophyseal extract or isolated neurohypophyseal hormones are added to the anatomical inside of the epithelia, the short-circuit current is increased, often by more than 100 %, and so is the rate of net Na transfer. Depending on the dose and other experimental conditions the stimulation of the active Na transport may last for anywhere between half an hour and several hours. In the guinea-pig caecal mucosa the response to neurohypophyseal extract is less pronounced and always of very short duration.

### *What is the mechanism of the Na transport ?*

In short, the similarities between these different cases of Na transport are sufficiently many to warrant the hypothesis that the underlying mechanism is the same and possibly also identical with the one active in the Na reabsorption in the kidneys. Current thinking concerning active transport is centered around the carrier hypothesis even in the case of active Na transport, and the present author also feels that it is by far the most attractive one. So far the evidence is mainly indirect. It is hard to conceive of anything but a chemical binding to a carrier molecule which is specific enough to transport Na (and to a certain extent also Li,) but not the other alkali metal ions (K, Rb and Cs). In case one wanted to explain the results in terms of the fluid circuit hypothesis, one might postulate, however, that the stream of solution going inward through the wide pores takes with it all the alkali metal ions, but that the smaller alkali metal ions like K and Rb are returned to the outside solution together with the excess solvent through pores which are too narrow to allow the big alkali metal ions, Li and Na, to pass through.

### *How can solvent flow be brought about ?*

Before proceeding further it may be appropriate to discuss briefly the mechanisms which may give rise to solvent flow through the pores of a membrane. The three most important ones are probably : 1) electroosmosis; 2) metabolically maintained concentration differences within the membrane phase and 3) osmotic pressure differences between the two media in contact with the membrane.

Electroosmosis has been discussed thoroughly for instance by Höber (7). The direction of flow is determined by the p.d. across the membrane, the fixed charges in the pores as well as by the valencies and dimensions of the ions present.

That metabolically maintained concentration differences within the membrane phase can bring about flow of solvent is felt intuitively. In a concise mathematical way systems of this type have been discussed by Franck and Mayer (4).

### *The nature of osmotic water flow*

Some confusion seems to exist in the literature with respect to the nature of the water movement during osmosis. It was stated by Jacobs (9) as early as in 1935 that osmosis and diffusion are different in principle although the two phenomena show formal similarities. Other workers have, however, treated osmosis as differential water diffusion. Thus the net water flow would arise as the difference between the amount of water diffusing in and that diffusing out, influx and outflux of water being proportional to the water activities (or concentrations) in the outside and inside media, respectively. In mathematical form :

$$M_{(w)\,in}/M_{(w)\,out} = a_{(w)\,o}/a_{(w)\,i} \tag{3}$$

or,

$$\frac{M_{(w)\,in} - M_{(w)\,out}}{M_{(w)\,in}} = \frac{a_{(w)\,o} - a_{(w)\,i}}{a_{(w)\,o}}$$

which can be written :

$$\frac{\Delta_w}{M_{(w)\,in}} \simeq \frac{c_{solutes}}{C_{water\,(o)}} \tag{4}$$

where $c_{solutes}$ is the difference in molar concentration of osmotically active particles between the media, and $\Delta_w$ is the net water transfer. $C_{water\,(o)}$ is the molar water concentration in the outside medium (55.6 moles/l.). The inadequacy of this equation to discribe conditions in a living membrane system was first pointed out by Hevesy, Krogh and Hofer (5) who made simultaneous determinations of net water uptake, $\Delta_w$, and heavy water diffusion ($M_{(w)\,in}$) into frogs, sitting in water. The ratio $\Delta_w/M_{(w)\,in}$ was between 3 and 5 times higher than calculated from the osmotic concentrations of the blood and the medium. Incidentally, Visscher *et al.* (20) also found a very poor agreement between experiment and values calculated according to equation *4* in a study of net water transfer and heavy water diffusion in the intestine. These authors, however, took the lack of agreement between theory and experiment as evidence for active water transport through the gut wall.

In our laboratory similar experiments where performed on isolated toad skin (Koefoed-Johnsen and Ussing, 10). Net water flow was measured by the volume change of the bathing solutions, whereas water diffusion was measured with $D_2O$. It was found in agreement with earlier observations by Krogh that no net water transfer takes place if the outside medium is made isotonic with

a non-penetrating substance like sucrose. Thus the water transfer observed with hypotonic outside solutions must be true osmotic water transfer. Nevertheless the discrepancies between theory (as expressed by equation *3*) and experiment was even greater than in the frog experiments of Hevesy *et al.* (5).

Especially in cases where the osmotic uptake of water was stimulated by antidiuretic hormone, $\Delta_w/M(w)\,in$ was higher, often as much as 16 times higher, than $\Delta c_{solutes}/c_{water\,(o)}$. If, however, it was assumed that the diffusing molecules of water like all other molecules present in the solution were speeded up when going in the direction of osmotic flow and slowed down when moving in the opposite direction, equation *2* would apply, and the results would be easily understandable. The very pertinent question then arises : does osmosis take place in the form of bulk flow of water? This has been contested recently, for instance by Chinard (2) who argues « that the osmotic pressure does not exist *per se* in a solution but is a pressure difference imposed by the analyst in the laboratory ». While this is obviously true, the situation is quite different when we are dealing, not with a solution, but with a system consisting of solution separated from pure solvent by a semipermeable pore membrane. For the sake of argument let us consider a single pore of finite length in a membrane which separates a sugar solution from pure solvent (compare figure 2).

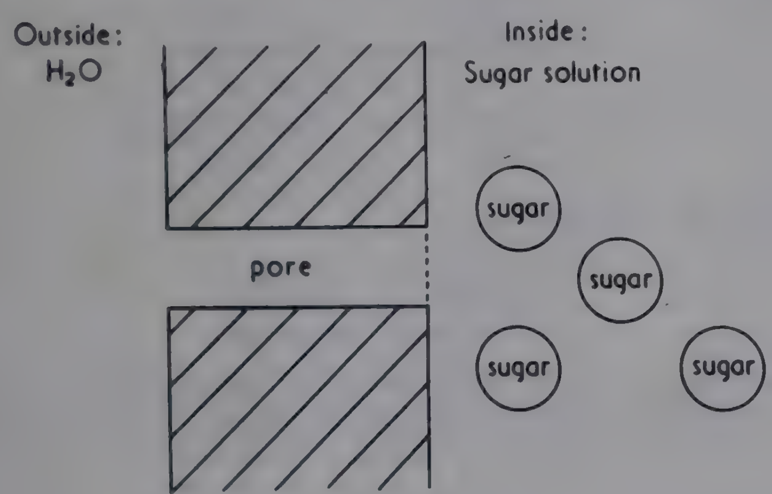


Fig. 2.

The sugar molecules which in the sketch are shown as spheres, are too big to penetrate the pore. The dotted line at the right hand end of the pore represents the level at which the sugar molecules are stopped. This boundary then in effect has the function of the physically ideal semipermeable membrane. Across this boundary, therefore, water molecules diffuse out of the pore and into the pore according to equation *3*, and since the activity of the water is lower in the sugar solution than in the pure water adjacent to the dotted boundary, water will move across the latter into the sugar solution. But what force makes the water move from the left hand opening of the pore to the dotted line ? The pore contains pure water all the way through, so the driving force cannot be a difference in the chemical activity. Obviously the driving force is the 'suction' created by the osmotic pressure difference at the dotted line. But suction is only another word for hydrostatic pressure difference. The movement of water through the length of the pore therefore is governed by the laws for laminar flow (for cylindric pores Poiseuilles law) and not by the laws of diffusion (Fick's law). For very narrow pores the deviations from the behaviour predicted by equation *1* will be unimportant and when the pores are of similar dimensions to the water molecules, the distinction between flow and diffusion vanishes. Since, however, laminar flow increases with the radius to the 4th power whereas diffusion increases only with the radius to the 2d power, the contribution of flow will soon become of decisive importance in the kinetics of water transfer. This explains that although the underlying mechanism of osmosis is one of water diffusion, still the kinetics is largely determined by the laws for bulk flow. For our present purpose it is important that osmotic pressure difference can give rise to bulk flow, which in turn creates solvent drag.

*Estimation of solvent drag in toad skin from water flux data*

The above mentioned experiments on the water movement through isolated toad skins thus can readily be explained on the basis of simple osmosis taking place through the pores of a semipermeable or nearly semipermeable membrane. It will be recalled from the beginning of this lecture that the drag force created by the flow of solvent can be estimated from the flux ratio of a suitable test substance (equation *2*). In the heavy water experiments, water itself may be said to have played the role of a test substance, the flux ratio being determined by the fact that the influx was speeded up and the outflux was slowed down by the bulk flow in the pores.

The following experiment with an isolated toad skin may be considered typical : the inside medium was Ringer and the outside medium 1/10 Ringer. The influx as estimated from the rate of diffusion of heavy water was 460 µl./cm²/h., whereas the net water flux was 13 µl./cm²/h. (determined by volume change). Thus the outflux must have been 447 µl./cm²/h. From this we obtain the flux ratio 460/447 = 1.03.

After addition to the inside solution of one international unit of neurohypophyseal extract the influx rose to 551 µl./cm²/h. whereas the net flux was found to be 36 µl./cm²/h. Thus the flux ratio was 551/515 = 1.07.

On the basis of the pore membrane theory the effect of the hormone must have been to increase the pore size, thus increasing the linear rate of flow and the solvent drag force.

In case all pores were of equal size, the water flux data might be used for the calculation of the solvent drag exerted on the Na ion. According to equation *2* we have :

$$RT \ln \frac{M(Na)\,in}{M(Na)\,out} = \frac{RT}{D_{Na}} \cdot \Delta_w \int_0^{X_0} \frac{1}{A}\,dx \qquad (5)$$

and :

$$RT \ln \frac{M(w)\,in}{M(w)\,out} = \frac{RT}{D_w}\,\Delta_w \int_0^{X_0} \frac{1}{A}\,dx \qquad (6)$$

Combining equation 5 and 6 we obtain :

$$RT \ln \frac{M(Na)\ in}{M(Na)\ out} = RT \frac{D_w}{D_{Na}} \ln \frac{M(w)\ in}{M(w)\ out} \qquad (7)$$

At 17.5° C. the self diffusion coefficient for water is according Rögener (14) $D_w = 1.77 \times 10^{-5}$, whereas the diffusion coefficient for Na is $1.075 \times 10^{-6}$ cm²/sec.

In case we want the drag force on the Na ion in electrical units (volts), we have to divide by 96 500 (Faradays number) :

$$\text{Drag force} = \frac{D_w}{D_{Na}} \cdot \frac{RT}{96\,500} \ln \frac{M(w)\ in}{M(w)\ out}$$

or, at 20° C. :

$$\text{Drag force} = \frac{D_w}{D_{Na}} \cdot 0.058 \log_{10} \frac{M(w)\ in}{M(w)\ out} \qquad (8)$$

Insertion of the values found gives a drag force of 0.8 mV before, and 1.7 mV after treatment of the skin with antidiuretic hormone. Considering that the active Na transport resulted in a potential difference of 82 mV in the control period and 108 mV in the experimental period, besides overcoming the concentration difference between 1/10 Ringer and Ringer, the contribution originating in the drag force seems quite negligible.

### *Estimation of solvent drag in toad skin from thiourea flux data*

Water has, however, the disadvantage as a test substance that its molecules are so much smaller than the Na ion. As a consequence of this, water may be able to penetrate by routes which are inaccessible to Na. It is true that the rate of Na transport and presumably the passive Na permeability is increased when the skin is treated with antidiuretic hormone, just as the rate of osmotic water flow is increased by the hormone treatment. This observation certainly indicates that the water and the Na ions move in the same pores. But if there were a metabolically induced return flow of water through a set of narrow pores, the flux ratio for water and thus the calculated drag force on the Na ion would come out too low. In order to make an estimate of the maximum drag force which can be exerted on the Na ion it is necessary to use a test substance which has at least the molecular size of the hydrated Na ion. The test substance further has to be highly hydrophilic and not likely to be transported actively.

One additional requirement has to be fulfilled, namely that the test molecule shall be able to penetrate the skin. This last requirement puts severe limitations on the choice. Even urea diffuses through only with difficulty and the permeability to sugars is practically nil. Our first choice was thiourea. Its molecule is slightly larger than the hydrated Na ion so that the drag force exerted on it would be greater than that on the Na ion, and so that, furthermore, back transport through a set of narrow pores would be even less likely than in the case of the Na ion.

In addition thiourea offers the experimental advantage that it can be labelled with two alternative labels, namely $^{14}$C and $^{35}$S. Since these two isotopes can easily be determined separately in a mixture, it is possible to determine influx and outflux of thiourea simultaneous with a good accuracy.

The first point to be considered is : does the antidiuretic hormone affect the rate of diffusion of thiourea through the toad skin ? This point is important because the whole argument must be in error if the hormone should not increase the permeability to a test substance like thiourea. As a matter of fact, the effect of the hormone on the rate of diffusion of thiourea is dramatic.

Table IV shows a few examples of this. The thiourea concentration was 3 mM on both sides of the skin. The permeability coefficient $k$, is given for the direction

TABLE IV

*Effect of neurohypophyseal extract (one international unit per 20 ml. unit bathing solution) on the inward permeability coefficient ($k_{in}$) for thioura diffusing through the isolated skin of the toad* (Bufo bufo)

| Outside medium | | $k_{in} \times 10^4$ cm./h. | |
|---|---|---|---|
| | | before addition of hormone | after addition of hormone |
| R/10 | I | 12 | 56 |
| | II | 10 | 149 |
| R | III | 9 | 275 |
| | IV | 12 | 104 |

Inside bathing solution Ringer. Thiourea concentration on both sides : 3 mM.

outside → in, only. The diffusion rate in the opposite direction responded similarly. In two experiments the outside medium was 1/10 Ringer, whereas in the other two outside as well as inside medium was Ringer. The hormone makes the permeability to thiourea go up sometimes by a factor of 25.

Our primary problem is, however, the effect which an osmotically induced flow of water through the skin has on the flux ratio of our test substance.

Only a few figures, means of larger series, shall be given here because the experiments are still in progress. The results seem, however, quite conclusive.

With 1/10 Ringer on the outside and Ringer on the inside, the flux ratio of thiourea before treatment with antidiuretic hormone was 1.18 and after the treatment 1.40. This indicates that the molecules moving in the direction of water flow are speeded up and those going against the flow are retarded in agreement with the pore theory. With isotonic solutions on both sides of the skin the flux ratio for thiourea was 1.0 even after hormone application and independent of whether isotonicity of the outside medium was obtained by addition of sucrose or by the use of Ringer. Thus with no net water flow there was no drag force. This again means that there can be no appreciable return flow of water through

narrow pores, and neither can there be some special type of water secretion.

Inserting the experimental figures in the equation for the drag force (*8*) we find that the water drag is equivalent to 4.3 mV before, and 8.8 mV after the application of the hormone.

*Conclusion*

The conclusion thus is that a fluid circuit mechanism is not operative in the amphibian skin. The drag force arising as a result of osmotic water flow is, however, of sufficient magnitude to modify significantly the ionic movements and thus the potential arising as a result of the active transport proper. In this context it is of interest that Harris has observed an increase in the outflux of Na from muscle when water is moved osmotically from the cells (compare Edvards and Harris, 3).

In the isolated toad skin with Ringer on both sides, an osmotic flow of water in the outward direction (induced by addition of sucrose to the outside solution) lowers considerably the transport potential, $E_{Na}$, of the Na ion whereas the resistance to Na, $(R)_{Na}$, goes down. If the osmotic water movement is directed inward, $E_{Na}$ is usually also depressed somewhat, but the most striking result is a violent increase in $R_{Na}$. The whole problem of solvent drag on ions undoubtedly is rather involved and presents points of considerable interest. But the driving force for active ion transport must be sought elsewhere. This study may be considered as additional indirect evidence for the carrier theory. But whereas it has usually been assumed that the combination with carrier made the inorganic ions lipoid soluble and thus allowed them to pass the cell membranes, the present material suggests that the chemical processes leading to the active transport are taking place in water-filled pores. To prove this point much more experiments on different types of membranes and with different test substances are required.

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## Chemical control of ion movements during nerve activity

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(*Received* 9 *April* 1955)

Active ionic transport across membranes occurs in nervous tissue in rest as it does in all living cells and maintains the inequality of ionic concentrations between the cell and its environment. What is characteristic of conduction is a transient increase in permeability to sodium ions and a net influx of sodium ion during the rising phase of the action potential and an equivalent net efflux of potassium ion in the falling phase (1, 2, 3, 4 and 5). These transports are in the direction of the concentration gradients and it is evident that these gradients are the latent source of electrical energy which is controlled by the permeability state of the membrane. What is the mechanism of this control ? What are the chemical reactions and membrane alterations associated with conduction ?

Studies by Nachmansohn and his colleagues during the last two decades have shown that the acetylcholine system is essential for conduction. This result was obtained by investigations in which acetylcholine has been integrated into the metabolic pathways of the cell and in which the sequence of energy transformations during nerve activity has been established. Moreover, it has been possible to correlate in various ways chemical and electrical events associated with the elementary process by which bioelectric potentials are generated (6, 7). The picture which has emerged as to the rôle of acetylcholine may be briefly outlined (figure 1).

Acetylcholine is, in resting condition, present in an inactive form. It appears likely that the ester is bound to a protein or a lipoprotein. During activity, as is

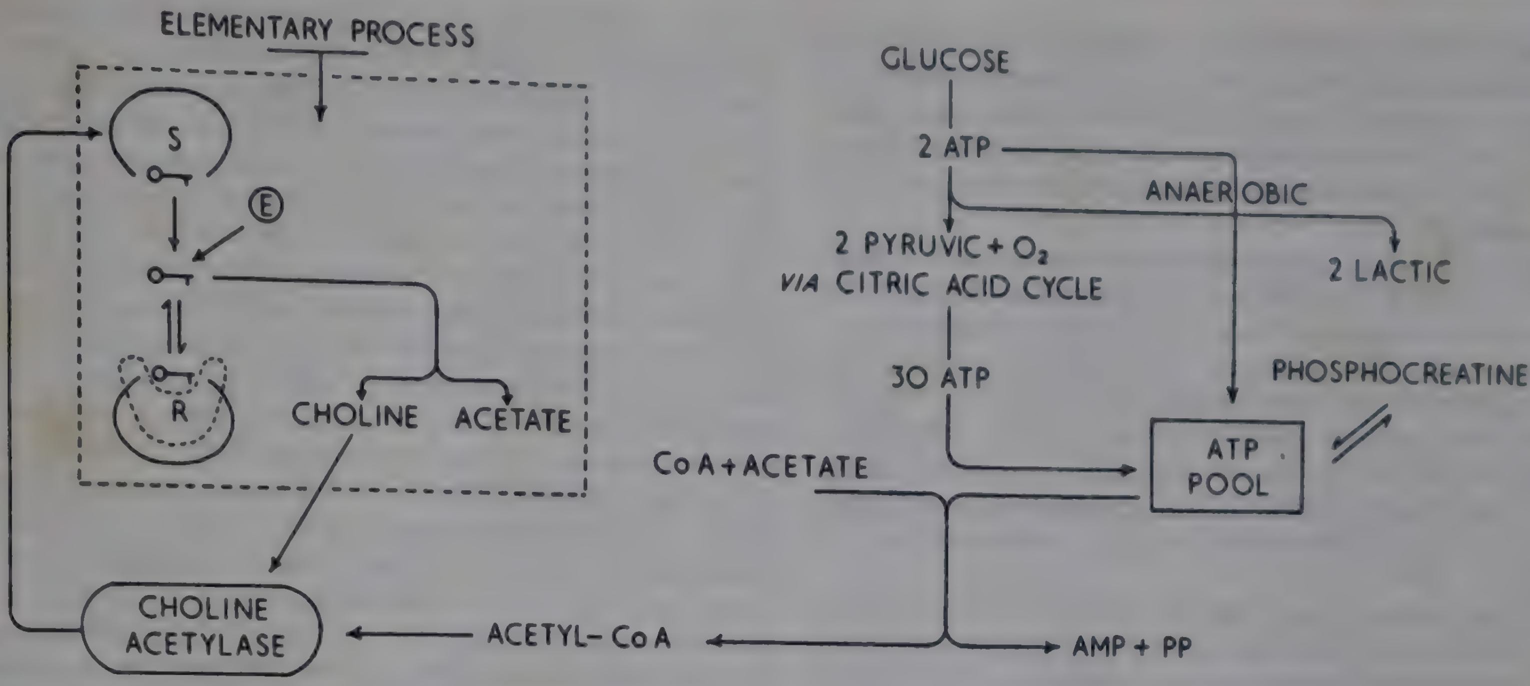


FIG. 1. — Sequence of energy transformations associated with conduction and integration of the acetylcholine system into the metabolic pathways of the nerve cell. The elementary process of conduction may be tentatively pictured as follows :

1) In resting condition, acetylcholine (represented by a key) is bound, presumably to a storage protein ($S$). The membrane is polarized.
2) *ACh* is released by current flow (possibly hydrogen ion movements) or any other excitatory agent. The free ester combines with the receptor ($R$), presumably a protein.
3) The receptor changes its configuration (dotted line). This process increases the Na ion permeability and permits its rapid influx. This is the trigger action by which the potential primary source of *EMF*, the ionic concentration gradient, becomes effective, and by which the action current is generated.
4) The combination between free ester and receptor is in dynamic equilibrium ; the free ester is, therefore, open to attack by acetylcholinesterase ($E$).
5) The hydrolysis of the ester permits the receptor to return to its original shape. The permeability decreases and the membrane is again in its original polarized condition.

well established experimentally, acetylcholine is released from the bound form and acts upon a receptor, the acetylcholine receptor. This action is responsible for the change of permeability and thus the generation of the electric potential. Although the acetylcholine receptor has not yet been isolated, the most likely assumption appears to be that it is a protein. Some data to be discussed later suggest that the effect of its reaction with acetylcholine may be a change in configuration of the protein.

The complex between acetylcholine and its receptor is in dynamic equilibrium with the free ester and the receptor. The free ester is subseptible to attack by the esterase and the hydrolysis will permit the receptor to return to its resting condition. The barrier for the extra influx of Na ion is thus reestablished. This action of the enzyme leads to the initial recovery and ends the cycle of the elementary process. The rapidity of the restoration process is made possible by the high speed of the enzymatic hydrolysis, which may occur in microseconds. Thus the nerve may respond to the next stimulus within a millisecond. The net result is the hydrolysis of acetylcholine and the exchange of Na and K ions. Complete recovery which need not be rapid requires the resynthesis of acetylcholine and the extrusion of Na ions.

A sharp distinction must be made between the active and recovery phases. The entrance of sodium and efflux of potassium during activity occur with the concentration gradients. The transient change in permeability requires little energy. This is only a trigger process making the potential energy effective. This view is supported by the extremely small amount of heat released during nerve activity, which is *e.g.* in the frog sciatic nerve of the order of magnitude of $10^{-11}$ of one small calorie per $cm^2$ per impulse. Fundamentally different is the situation with respect to the ion movements during recovery. The restoration of the original steady state requires extrusion of sodium against the concentration gradient. This process requires a relatively large amount of energy. Most of the extra heat produced by nerve activity is developed after the electrical changes and is presumably associated with the restoration of the electrolyte distribution. The energy for this 'active' ion transport must be assumed to be provided by the chemical energy derived from reactions common to most cells.

Knowledge of the forces of interaction between acetylcholine and the proteine of the system has become of paramount importance for an understanding of the elementary process. At present only the two enzymes are available in purified aqueous solution ; studies with the receptor must be made with intact structures, whereas nothing is known of the postulated storage protein. However, knowledge acquired with one of these proteins may aid us in understanding processes involved with the others. A molecule such as acetylcholine has only a limited number of features which may contribute to its interaction with proteins. All proteins of the system reacting specifically with the ester must do so through much the same elementary interactions and must, therefore, be similarly constituted at the active site. Small differences might be sufficient to lead to a considerable alteration of function.

The most suitable protein for studying the molecular forces responsible for interaction with acetylcholine is the esterase. Extensive investigations of the molecular forces have been carried out during the last few years by Wilson and a considerable amount of information has been obtained (8).

Investigations with appropriate substrates and inhibitors have revealed that there is a negative site in the enzyme surface which attracts the cationic nitrogen by Coulombic forces. In addition the alkyl groups of the nitrogen contribute to the binding by Van der Waal's forces. Studies with a series of competitive inhibitors show that except for the fourth alkyl group each methyl group increases the potency of an inhibitor by a factor of about 7. The fourth alkyl group contributes less to the binding.

Besides the anionic site which attracts the cationic nitrogen and helps in binding and orienting the molecule, there is also a group in the enzyme surface which reacts with the ester group and has been referred to as the esteratic site. The carbon of the carbonyl group has a strongly electrophilic character and forms a covalent bond with a basic group in the enzyme surface. Considerable information has been obtained from the pH dependence of the hydrolytic process catalyzed by the esterase. Mathematical analysis has revealed the presence of basic and acidic groups in the esteratic site. The basic group has a pH in the neighborhood of 6.5 and it is possible that this group is an imidazole derivative. In summary, the enzyme substrate complex as illustrated schematically in figure 2 is stabilized by Coulombic and Van der Waal's forces at the anionic site and by the covalent bond formation at the esteratic site. The two sites have been demonstrated to be functionally and spatially separate.

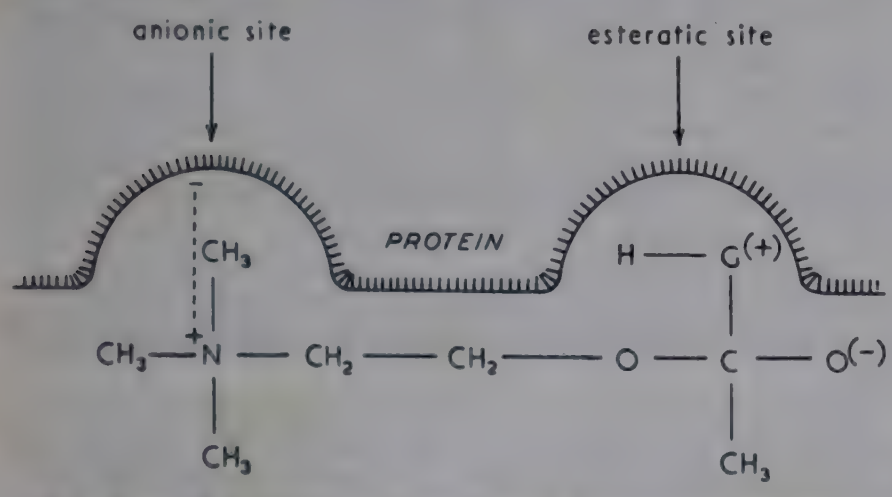


**Fig. 2.** — Schematic presentation of interaction between the active groups of acetylcholinesterase and its substrate.

The mechanism of hydrolysis has been shown to involve the formation of an acylated enzyme as an intermediate. The predictions of the theory have been borne out by experiments in various ways (8). The elucidation of this process has initiated many interesting developments. Among the many results may be mentioned the explanation of the mode of action of the 'nerve gases' the alkylphosphates. Whereas the action of these compounds has been considered for a long time to be irreversible, it became possible on the basis of theory to reverse the inhibitory action of these compounds (9, 10).

One feature of special interest in connection with the generation of bioelectric potentials is the different behavior of the proteins of the acetylcholine system to tertiary and quaternary nitrogen compounds. The bindings forces between the esterase protein and small molecules are, as mentioned before, not greatly affected by the presence of the fourth alkyl group, but the functional activities are markedly altered. The tertiary analogue of acetylcholine is split at less than half the rate of the splitting of acetylcholine itself (11). The difference is even more pronounced in the case of acetylase : removal of one methyl group reduces the rate of acetylation by choline acetylase by a factor of 12 (12). The biological activity of dimethylaminoethyl acetate upon the receptor measured by assay with frog's rectus abdominus muscle is only one hundredth of that of acetylcholine. It has long been known that the pharmacological action of quaternary compounds is much stronger than that of their tertiary analogues.

The striking effect of the fourth alkyl group upon the functional activity of the three proteins has been observed in many respects. This is all the more remarkable since the quaternary group is chemically saturated. The chemical reactivity, therefore, offers no satisfactory explanation. A clue may be the tetrahedral structure of the quaternary group. Such a structure is more or less spherical, and the only way the protein could be simultaneously in contact with all methyl groups would be by enveloping the molecule. This implies a change in protein configuration. A change of configuration would be of special interest with regard to the functional properties of the receptor. Rearrangement of acidic and basic groups by folding (or unfolding) of protein chains of the receptor would be one possibility to account for the increased sodium permeability effected by the system.

The existence of a receptor has long been postulated. Recent investigations have made it possible to separate experimentally the action upon the acetylcholine receptor from that upon acetylcholinesterase. This result was achieved by the development of two new techniques.

— Microtechniques have made possible the introduction of electrodes inside the cells, thus permitting direct measurements of the potentials across resting and active membranes. During the last two years such methods have been applied to the study of the transmembrane potentials of the electroplax of Electrophorus electricus (13, 14). It was found that a propagated spike similar to the action potentials of nerve and striated muscles may be elicited either by direct stimulation or by nerve stimulation. The penetration of quaternary nitrogen derivatives is sufficient to affect the active membrane of the electroplaque in contrast to axons or striated muscles. It offers, therefore, a favorable material for studying the difference between the action of tertiary and that of quaternary compounds upon the electrical potentials of the cell.

— Whereas in studies of the enzyme proteins in solution the reaction of one components is followed, in intact cells all the proteins are present in close vicinity. The active tertiary and quaternary nitrogen derivatives are structurally related to acetylcholine and therefore they will have affinity to both receptor and esterase.

Since binding to either member of the system will in theory block the propagated spike, it is essential to be able to distinguish between these two different causes of the electrical failure which follows upon the application of these compounds. This difficulty has been overcome by the development of a procedure for measuring esterase activity of the intact electroplaque at the time when failure of the propagated electrical potentials occurs (15).

In some cases electrical activity fails when the esterase activity has been barely affected, in others when it is still higher than 20 per cent of normal, the critical value which has been found in a number of studies with other materials; but in some when the enzyme activity is less than the minimum required. In those cases in which the enzyme activity is greater than 20 to 30 %, block of electrical activity is attributed to combination with the acetylcholine receptor (table I).

The affinities of decamethonium, carbamylcholine, and procaine for the esterase is very much smaller than that of eserine or prostigmine. The dissociation constants of the enzyme complexes of the first group are about $10^{-4}$ M, whereas those of the latter two drugs are about $10^{-7}$ M. On this basis it might have been anticipated, as is now demonstrated, that the esterase would not be

TABLE I

*Relationship between the effects of some compounds on the conducting membrane of electroplax and on the hydrolytic activity of intact cells*

| Compound | Concentration µg./ml. | % remaining activity |
|---|---|---|
| *non-depolarizing* : | | |
| T.P. | 500 | 9 |
| T.P. | 1 000 | 4 |
| Eserine | 500 | 28 |
| *d*-Tubocurarine | 700 | |
| Procaine | 500 | 91 |
| Procaine | 100 000 | 51 |
| DFP | 300 | 10 |
| *depolarizing* : | | |
| Prostigmine | 300 | 51 |
| Decamethonium | 10 | 86 |
| Decamethonium | 2 000 | 60 |
| Carbamylcholine | 10 | 99 |
| Carbamylcholine | 200 | 70 |
| Carbamylcholine | 2 000 | 83 |

The electroplax were taken from the Sachs organ of *Electrophorus electricus*. All the agents used block propagation, some with and some without depolarization. Ethyl monochloroacetate was used as substrate in 0.03 and 0.01 M concentration. Segments of 6 cells were exposed to the compounds for about 30 min. before addition of substrate from sidearm. The smallest concentrations employed are those which cause block of propagation in that period of time. 25° C. pH 7.6 (15).

greatly inhibited at the low concentrations (externally applied) necessary to produce block with decamethonium and carbamylcholine. Although in the case of the tertiary analogue of prostigmine, T. P., the affinity for the esterase is relatively low, block evidently may be caused by enzyme inhibition. As will be seen later, the receptor is also greatly affected.

Knowledge concerning different types of interactions with the enzymes may be transferred very readily to interactions of small molecules with the receptor and thereby clarify our concepts. Substances interacting with the esterase conveniently fall into the two classes, substrates and inhibitors. The inhibitors which yield the most significant information are those which react with the same sites as the normal substrate, *i.e.* competitive inhibitors. But even for these inhibitors the structural requirements are much less restrictive than for substrates. To be an inhibitor, a molecule need only be bound by the enzyme, but to be a substrate the molecule must be so constituted that the binding is attented by simultaneous or subsequent changes of extensive consequences. In this connection it is important to recognize that a moderate decrease, say 10-fold, in binding can always be compensated by utilization of higher concentrations. It is true that binding plays an important part in the determination of enzyme specificity in physiological function, but in an experiment the concentrations are under the control of the investigator. But the specific functional activity cannot be controlled and here the special structural requirements are dominant. For this reason a tenfold difference in activity is very great indeed. We therefore must compare differences of binding and differences of specific activity on different scales. We have already seen that the binding of a tertiary compound by the enzymes of this system (esterase) is not very different from the binding of the analogous quaternary compound, but that the specific activities of tertiary compounds are significantly weaker. We might consider one further case which emphasizes that binding is a preliminary and less specific prerequisite to activity. Similar views were expressed by Clarke (16). Butyrylcholine is somewhat better bound by the esterase than acetylcholine, yet it is hydrolyzed less than 1 % as rapidly. Evidently the larger acyl chain very seriously interferes with secondary processes which bring about the hydrolysis.

Conductive tissue is designed to propagate an impulse, or more specifically to propagate a transient change in transmembrane potential. The specific activity which we are considering in this report are those changes in the acetylcholine receptor which lead to or produce depolarization of the cell membrane. The change in transmembrane potential is the indirect experimental sign of changes in the receptor. Interference from enzyme activity tells us that binding is a less specific prerequisite to receptor activity and that in general we may expect to find a large number of substitued ammonium ions capable of forming receptor complexes. Only a few which meet more closely defined requirements will be receptor activators analogous to enzyme substrates, the rest will be receptor inhibitors. But both will block the propagated depolarization, for the one will hold the receptor in an inactive state and the other in an active

state. The former block without depolarization, but the latter block and also depolarize (table I).

It is possible, with these concepts based upon enzyme theory, to readily interpret the observations on the interaction of many drugs with the electroplaque and other electric tissue. Receptor activity occurs either simultaneous with or subsequent to the binding of a receptor activator. Since a receptor inhibitor reacts with the same receptor site as a receptor activator, it follows that if an inhibitor is applied it should prevent the activation of the receptor by the subsequent addition of an activator. The two are competitors so that the effect of one should be overcome by increasing the concentration of the other. Thus it is found that the depolarization caused by the receptor activator, carbamylcholine is antagonized by procaine, eserine, *d*-tubocurarine, and the tertiary analogue of prostigmine (T.P.), all of which must therefore be recognized as receptor inhibitors. These observations confirm our explanation of the blocking action of these compounds.

In the case of TP and possibly eserine, the enzyme activity has been reduced below the critical value and it is therefore clear that electrical block can be thereby explained, but it is also apparent that the receptor has been inhibited and this also is sufficient to produce block. Presumably, as the compounds diffuse into the cell membrane first one protein and then the other is reduced below a critical level depending upon their relative affinities for the compound, but our present methods are not in this case sufficiently sensitive to freeze the system with only one protein critically inhibited.

Studies of the contracture of the abdominus rectus muscle elicited by acetylcholine and inhibited by eserine were interpreted by Kirschner and Stone (17) in terms of competitive inhibition of the receptor.

We now come to the question : « what structural features endow a molecule with receptor binding properties ? » Evidently very much the same structures that interact with the esterase also make for interaction with the receptor. These have already been mentioned. If we make the reasonable assumption that a fair fraction of the receptor is in the form of a complex at the time of receptor inhibition or receptor activity, it follows from the low concentrations of drugs necessary to block electrical activity or in general to elicit pharmacological response, that the binding of some of these compounds to the receptor is considerably stronger than to the esterase.

It appears reasonable to suppose that the combination of the receptor with each of these compounds obeys the law of mass action and that there is in addition to a binding constant a maximum receptor activity for each compound (small or zero for receptor inhibitors) corresponding to saturation of the receptor and analogous to the maximum velocity of an enzyme and a substrate. It would be most revealing if we could treat our observations so as to measure separately the binding constant and the maximum activity, but there are many obstacles to this type of experimentation. First, we have no assurance that the extent of depolarization is proportional to the extent of binding and secondly, we do not know the concentration of the compound in the active membrane. The consequence is that the comparative studies which have been made contain unknown contributions from penetration rates, binding constants, and maximum activities. Even so certain general rules for receptor activity have emerged. The quaternary structure, as already mentioned, is very important and it should be highly methylated. In addition an electrophilic carbon atom such as carbonyl carbon atom enhances activity. A complete review of the effect of various compounds and the influence of structure at the neuromuscular junction is given by Riker, W. F., Jr. (18).

The purpose of this paper is to describe how certain diverse effects of drugs can be interpreted in terms of interaction with an acetylcholine receptor and to describe how the receptor may function as part of a system, the acetylcholine system including besides the receptor, acetylcholine, acetylcholinesterase, choline acetylase and a storage protein. The rôle of the acetylcholine system in the generation of bioelectricity has been described in so far as present knowledge permits.

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# The effect of antibiotics on the nutritional requirements of animals

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(*Received* 4 *April* 1955)

The effect of antibiotics in increasing the growth of animals has been well established and a great deal of effort has been directed towards determining the mechanism of this response. It soon became evident that this growth is not the result of the antibiotic entering the metabolism of the animal and serving as precursors of essential metabolites. The activity of various antibiotics of widely varying chemical structure precluded this possibility. Also the inactivity of antibiotics for chicks in a germ-free environment (1) and the failure of chlortetracycline to stimulate the developing chick embryo (2) supports the view that the effect is on the intestinal microflora. It then becomes of interest to know how a modification of these microorganisms can increase growth of the animal? Since the intestinal flora had previously been shown to synthesize vitamins, it was natural to inquire into the effect of antibiotics on vitamin syntheses. Many experiments have been made to explore this relationship, and evidence for a vitamin sparing effect has been obtained. This discussion will deal with the effect of antibiotics on vitamin requirements and on the synthesis of these vitamins by intestinal microflora.

It should be noted that while a vitamin sparing action can explain in part the growth response on diets marginal in vitamins it does not account for the growth observed in nutritionally complete rations. It should also be emphasized that the ability to get a growth response with either an antibiotic or a vitamin on a suboptimal diet does not establish a vitamin sparing effect. The only true criterion is a reduction in the vitamin requirement as measured by a dosage response curve or a greater response to an antibiotic on a suboptimal level than on an optimum level of the vitamin.

### *Effect of antibiotics on requirement of water soluble vitamins*

The possible relationship between antibiotics and vitamin $B_{12}$ was suggested by a report that chlortetracycline and combinations of antibiotics would produce hematological responses in pernicious anemia (3). This, together with the observation of Davis (4) that *E. coli* could absorb vitamin $B_{12}$ from solution, suggested that antibiotics might increase the availability of dietary vitamin $B_{12}$ by preventing the uptake of this vitamin by *E. coli* which would render it unavailable to the host. However, this inviting explanation did not find support in subsequent experiments.

The effect of chlortetracycline on vitamin $B_{12}$ requirements of the chick was determined by feeding graded levels of the vitamin in the presence and absence of the antibiotic (5). In five out of eight experiments the response to chlortetracycline was greatest at suboptimal levels of vitamin $B_{12}$. Thus, chlortetracycline produced a growth response of 43 % in a deficient ration containing 2 μg./kg. of vitamin $B_{12}$ and of only 3 % on an adequate diet containing 50 μg./kg. of vitamin $B_{12}$. In other experiments, vitamin $B_{12}$ had no effect on the growth response to chlortetracycline. Although chlortetracycline was without effect on vitamin $B_{12}$ requirements in some experiments it did have a marked effect in reducing the high mortality occurring on the vitamin $B_{12}$-deficient diet. The average mortality, in eight experiments, on vitamin $B_{12}$-deficient diets was 48 % in the absence of chlortetracycline and 16 % in its presence. This reduction in mortality occurred even where there was no evidence of a vitamin sparing effect. Similar results have been obtained in other vitamin and in amino acid deficiences, and suggest that the mortality resulting from these deficiency states may be partly the result of bacterial invasion which is minimized by antibiotic feeding.

The effect of antibiotics on vitamin $B_{12}$ was also measured on a methionine-deficient ration containing homocystine. On this diet the vitamin $B_{12}$ requirement is greatly increased because of the increased demand of this vitamin for the methylation of homocystine. Chlortetracycline did not show any evidence of a sparing effect on this highly vitamin $B_{12}$-deficient diet.

The vitamin-sparing effect has also been studied by the effect of antibiotics on hatchability. Vitamin $B_{12}$ is essential for the development of the embryo and hatchability will decrease to less than 20 % on deficient diets. Peterson *et al.* (6) found that 0.75 μg. of vitamin $B_{12}$ per kg. of diet increased hatchability from 15 up to 58 % while 50 mg. of streptomycin or chlortetracycline were without effect. While antibiotics showed little effect in giving a vitamin $B_{12}$-like response on a highly

deficient diet, they have been found to exert a significant carry-over effect on the progeny. Bentley and Hershberger (7) observed a significant increase in the growth of chicks when bacitracin or chlortetracycline were included in the maternal diet. This was most marked when both the maternal and the chick diets were deficient in vitamin $B_{12}$. This suggests that the carry-over effect was the indirect result of an increased synthesis of this vitamin. Assays of the day-old chick livers revealed no increase in vitamin $B_{12}$ content as a result of antibiotic addition to the maternal diet, while the addition of vitamin $B_{12}$ itself produced 6 to 10-fold increases. Small increases in the vitamin $B_{12}$ content of the egg yolks were observed. On the basis of the small increases in vitamin $B_{12}$ content of the egg yolk and the day-old chick livers, it seems difficult to account for the carry-over effect on the basis of an increased synthesis of vitamin $B_{12}$.

Other experiments have also indicated that under certain conditions antibiotics may increase progeny growth performance (8, 9). In a study with baby pigs on a synthetic milk diet, vitamin $B_{12}$ produced a 39 % growth increase in the absence of an antibiotic but had no effect in the presence of chlortetracycline (10). This antibiotic produced a 77 % increase in growth which was the same as that given by a combination of chlortetracycline and vitamin $B_{12}$. This sparing effect was not observed with streptomycin, and vitamin $B_{12}$ produced a response in the presence of this antibiotic.

Wahlstrom and Johnson (11) used baby pigs on a synthetic milk diet and studied the hematological response to vitamin $B_{12}$ and chlortetracycline. The reduction in reticulocytes which developed on this diet was not cured or prevented by the feed of chlortetracycline. Parenteral administration of vitamin $B_{12}$ to the animals which had received chlortetracycline for 7 weeks produced a marked reticulocyte response in two animals. In another experiment two baby pigs which had been depleted of vitamin $B_{12}$ for 39 days were given 100 ppm of chlortetracycline. The reticulocyte count continued to fall and there was no evidence of vitamin $B_{12}$-like response to the chlortetracylcine. The synthetic milk diet employed by Wahlstrom and Johnson (11) contained purified soybean protein and was, therefore, much more deficient than that of Sheffy *et al.* (10) which contained casein.

In the rat, antibiotics increase the bacterial synthesis of vitamin $B_{12}$ in the intestine, but this does not always appear to be available to the animal. The feeding of chlortetracycline increased the amount of water soluble vitamin $B_{12}$ in the cecum and the colon but not in the ileum. The absence of an increased concentration of vitamin $B_{12}$ in the liver and kidney indicated that the vitamin $B_{12}$ was not absorbed. The feeding of small amounts of vitamin $B_{12}$ had little effect on its concentration in the intestine but did produce five to fourteen-fold increases in kidney and liver.

By the use of radioactive cobalt (12) it was found that chlortetracycline increased the amount of vitamin $B_{12}$ in the feces by three to eight-fold. However, examination of the tissues for radioactivity indicated that very little vitamin $B_{12}$ had been absorbed. This failure to utilize the increased vitamin $B_{12}$ synthesized in the presence of antibiotics may be due to production of « *pseudo* »-vitamin $B_{12}$ which is microbiologically active but inactive for animals. It is also possible that the vitamins synthesized in the lower part of the intestinal tract are not absorbed and, therefore, are not available to the animal. Similar results have been obtained with chicks (13) where the addition of either succinylsulfathiazole, streptomycin, penicillin or bacitracin to a dextrincontaining diet did not affect the vitamin $B_{12}$ content of the livers. There was also no effect on the pantothenic acid, pyridoxine or niacin contents of the liver, but there were two to five-fold increases in folic acid and citrovorum factor.

The most consistent vitamin sparing effect of antibiotics has been observed with thiamine in rats (14, 15). This effect varies with the type of carbohydrate in the diet, being greater with dextrin than with sucrose. In these studies antibiotics gave no response when adequate quantities of the vitamins were present. Large growth increases were obtained on suboptimum thiamine levels. Thus, on a thiamine-free diet the gain in 28 days was 12 g. in the absence of and 89 g. in the presence of penicillin. At intermediate thiamine levels, rats receiving 1 p.p.m. of thiamine gained 61 g. in the absence of and 112 g. in the presence of this antibiotic. Two ppm of thiamine produced a gain of 102 g. which was not further improved by penicillin. Different antibiotics vary in their thiamine sparing ability. Penicillin proved to be the most effective (15, 14, 16), streptomycin was less active, chlortetracycline and oxytetracycline were inactive and chloromycetin appeared inhibitory (15).

Riboflavin requirements were less effected by antibiotics than those of thiamine. Chlortetracycline and penicillin gave no response on a riboflavin-free sucrose-containing diet. At intermediate levels of riboflavin, chlortetracycline and penicillin were equally effective in promoting growth. Penicillin, chlortetracycline, streptomycin and oxytetracycline were equally effective in promoting growth on pantothenic acid deficient diets (16, 15).

The growth response to penicillin on a thiamine-free diet indicates that the antibiotic must increase intestinal synthesis. The response at intermediate levels of vitamins might be explained by a suppression of organisms which absorb vitamins and thus make them unavailable to the host. Such an explanation is not possible on thiamine-free diets and any thiamine sparing effect must be the consequence of increased bacterial synthesis. This view is supported by the observation that penicillin gives a growth response irrespective of whether the limiting vitamin is injected or fed (17). Assays of the contents of the small intestines revealed greater quantities of thiamine in those animals receiving penicillin. Experiments using oral radiothiamine to study synthesis or absorption of the vitamin in the presence and absence of penicillin support the hypothesis that there is an increased intestinal synthesis in the presence of the antibiotic.

Inhibition of organisms which inactivate or render vitamins unavailable to the host has been suggested as been suggested as one explanation for the nutritional effect of antibiotics. Support for such a view is found in observations of de la Huerge and Popper (18) that two-thirds of an orally administered dose of choline is excreted as trimethylamine. This appears to be the

result of bacterial action as the administration of penicillin or chlortetracycline reduces the conversion trimethylamine. The effect of antibiotics is only temporary, lasting for a few days. It is known that administration of antibiotics temporarily reduces the bacterial population but that after several days it returns to its original level. The new organisms that re-establish themselves in the presence of the antibiotic apparently possess the same ability to inactivate choline as did the original.

The feeding of live yeast is known to absorb thiamine from the intestinal tract and thus precipitate thiamine deficiency in the animal (19). *E. coli* is also known to absorb vitamin $B_{12}$ from solution (4) but there is no evidence to indicate that this effect can take place in the digestive tract and thus make vitamin $B_{12}$ unavailable to the host. This is shown by the fact that feeding Sulfamethazine, which inhibits growth of *E. coli*, does not modify the vitamin $B_{12}$ requirements (5).

Makino and Umezu (20) found that the indole production in the intestine was decreased by the feeding of oxytetracycline to rats. In the absence of the antibiotic the indole content of the intestinal contents increased from the duodenum to the colon. In the treated animals, the indole content was the same in different parts of the intestinal tract. Bacteria isolated from control animals were more active in the formation of indole than those obtained from animals treated with oxytetracycline. This formation of indole may represent a significant loss of tryptophane.

### *Antibiotics in hepatic and renal disorders*

Antibiotics have an effect on liver and kidney lesions which have been induced by dietary procedures. Gyorgy *et al.* (21) found that rats receiving large amounts of yeast died with liver necrosis within 30 to 40 days. This necrosis could be prevented by feeding either methionine, cystine or vitamin E. It could also be greatly reduced and delayed, but not prevented, by feeding 5 to 25 mg. of chlortetracycline per day. This would correspond to 800 to 4000 ppm in the diet.

The effect of chlortetracycline in preventing liver necrosis became progressively less in successive experiments and finally disappeared after one year. At this time new experiments which were started in other laboratories with the same strain of rats showed the protective effect of chlortetracycline. When the original laboratory and experiment was thoroughly disinfected the protective action of antibiotics against liver necrosis was again observed. The question was raised whether the ability of antibiotics to delay, but not to completely prevent, the development of liver necrosis might be the result of development of resistant organisms. This possibility was explored by starting the experiment with one antibiotic and then switching to another at 44 days. Since there is no cross resistance between penicillin and chlortetracycline, this switching procedure should increase the effectiveness of the antibiotic in delaying necrosis. The results showed that changing from chlortetracycline to penicillin or *vice versa* was no more effective than either antibiotic alone continued throughout the entire experiment. This strongly suggests that the development of resistance does not play an important part in limiting the effectiveness of antibiotics in the prevention of massive hepatic necrosis (22).

The possibility has been considered that liver necrosis is the result of toxin production by intestinal bacteria (23). However, Gyorgy *et al.* (23) were unable to correlate the action of chlortetracycline, penicillin and oxytetracycline with any change in the composition of the intestinal flora (24). It was also impossible to demonstrate any hepatoxic agent in the cultures of fecal bacteria or *E. coli*. Further attempts were made by Luckey *et al.* (25) to determine the effect of the intestinal flora on liver necrosis by the use of germ-free techniques. They reported that rats fed on a necrogenic diet and grown under germ-free conditions did not develop liver necrosis when they were fed *ad libitum*. The feed consumption of the germ-free animals was approximately double that of normal controls. However, the germ-free rats did develop necrosis when their feed consumption was restricted to that of the conventional controls. The finding that the feed consumption in germ-free rats is higher than normal controls is in line with the observation that antibiotics frequently increase feed consumption and suggests that some of the physiological effects produced by antibiotics are the results of increased feed intake.

Antibiotics are also effective in the prevention of renal lesions which develop on high fat-low choline diets (26). It had previously been found by these same workers that the incidence of renal damage was greater on diets containing sucrose than on those containing starch. This suggested the role of the intestinal flora on this disorder. The incidence of renal damage in the control animals on a sucrose diet was 78 %. The feeding of vitamin $B_{12}$ reduced this to 46 % and choline completely prevented therenal lesions. Chlortetracycline at a level of 5000 p.p.m. reduced the incidence to 20 %, and chloromycetin and oxytetracycline had essentially no effect. Analysis of the liver and kidneys of the chlortetracycline-treated animals revealed small increases in choline but these appeared insufficient to account for the effect on the basis of an increased intestinal synthesis of choline. There was no significant increase in choline in the feces on the feeding of antibiotics. It was not possible to account for the protective action of chlortetracycline on the basis of synthesis of either choline or vitamin $B_{12}$.

A similar lipotropic effect was observed in dogs by Kaplan *et al.* (27). They observed that dogs with ligated pancreatic ducts, or which have been depancreatized and are receiving insulin, develop fatty livers, a reduction in blood lipids and an elevation in serum alkaline phosphatase. Lipotropic agents such as choline restored liver fat and blood lipids to normal. Administration of 1.0 g. of chlortetracycline per day produced an increase in ester cholesterol and a reduction in alkaline phosphatase to normal. The effect reached a maximum in three weeks and then returned to 50 % of the peak value. The effect was not due to a suppression of bacterial conversion of choline to trimethylamine as there was no consistent depression of choline destruction during chlortetracycline feeding which was large enough to account for the lipotropic effect.

*Antibiotics and fat soluble vitamins*

Antibiotics have been reported to have an effect on the utilization of fat soluble vitamins. Burgess *et al.* (28) found that feeding penicillin to chicks increased the carotenoid level in the serum and the vitamin content of the liver. Similar effects on serum carotenoid levels were observed by Squibb *et al.* (29) on the feeding of chlortetracycline to laying hens. Barber *et al.* (30) found that penicillin increased the vitamin A content of the liver by 50 %. It appears that the antibiotic may be more directly involved in the absorption of carotene or its conversion to vitamin A. High (31) observed that chlortetracycline produced significant increases in liver storage in the rat when carotene was fed but not when vitamin A was used. Chlortetracycline also increased the availability of vitamin A by an average of 24 % as measured by vaginal smear assays with rats (32). The effect was observed even when the antibiotic was given 24 hours after the administration of the vitamin A, which shows that the antibiotic did not exert its effect solely on vitamin absorption. The antibiotic did not increase liver storage of vitamin A which is in agreement with the results of Hartsook *et al.* (33).

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# Bacteriological implications of growth-stimulation by antibiotics in different environments (*)


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(*Received* 26 *May* 1955)

Although there is ample evidence, discussed by other contributors to this symposium, that antibiotics given in the diet may spare certain nutrients, it is apparent that some further mechanism of growth stimulation must be involved since antibiotics can, in certain circumstances, increase the growth of animals receiving a nutritionally adequate diet. In our own laboratory we have been unsuccessful in attempts to devise a diet that would support optimal growth in chicks in all circumstances without the addition of antibiotics. With practical rations containing adequate amounts of the known essential nutrients growth could be improved by inclusion of ingredients such as dried milk, raw liver or fish solubles which might be expected to supply unidentified

(*) N. I. R. D. paper nr. 1673.

growth factors, but the growth increases were not so great as those achieved by further addition of antibiotics.

In early experiments with chicks given adequate diets we showed (1) that their growth in new premises could not be improved by inclusion of procaine penicillin in the diet, whereas their hatch-mates in a room regularly used for chick-rearing grew submaximally unless they received penicillin. When chicks from the old premises were brought into contact with chicks from the new, the growth of the latter without antibiotics was reduced. These findings led to our suggestion that the antibiotic was eradicating a subclinical condition which prevented maximal growth but was otherwise inapparent.

Other workers have since confirmed our findings with chicks (2, 3) but the growth-depressing condition has not so far been characterized. We were able to eradicate it temporarily from chick rooms by fumigation and disinfection followed by a period when the rooms were left empty; the first birds introduced into the premises after such treatment did not respond to penicillin but, in spite of a stringent hygienic routine including periodic fumigation and daily disinfection, as further batches of chicks were reared there, growth in the absence of penicillin deteriorated (4). Our experience was similar in field conditions. Chicks in new fold units on fresh grassland responded only slightly to penicillin in the diet. The folds were scrubbed with lysol and moved to fresh pasture between each successive batch of birds, but as they continued in use a small but significant response to penicillin was obtained (4).

The ease with which the growth-retarding condition re-established itself in our laboratories and elsewhere (2) made impossible a closer study of its nature in ordinary chick-rearing accommodation. In order to investigate it further we constructed small isolation units of transparent plastic (5). In each of these units two groups of five chicks, one with and one without penicillin, were reared to 2 weeks of age in complete isolation from other birds. In such conditions no growth response to penicillin was observed. When the chicks in the isolation units were allowed to eat gut contents taken from birds in the room where growth was improved by antibiotics, the weight attained at 2 weeks of age was less in those birds not receiving penicillin. No such effect was observed when the gut contents were autoclaved, or when they were taken from birds in new premises where no growth increase could be brought about by penicillin.

Cooper and Gordon (6) were unable to demonstrate any response to penicillin in their premises until the growth-retarding condition was deliberately introduced; this was done by giving in their chick diet small quantities of gut contents taken from birds reared in our own laboratories where dietary penicillin increased growth. Once the condition was established Cooper and Gordon found it possible to transmit it, either artificially by giving small quantities of gut contents or by rearing fresh chicks in the same room as the affected birds. The fact that the condition could be transmitted thus without direct contact between birds suggested to these workers that the causative agent(s) might be airborne, and in an investigation of methods of transmitting the condition they found that when given either by mouth or intranasally to new chicks crude filtrates of lung tissue from affected birds caused a depression in growth counteracted by penicillin.

Several workers have tried to correlate alterations in the growth rate of chicks with bacteriological changes. Romoser, Shorb, Combs and Pelczar (7) observed gram-positive non-sporulating rods in the caeca of chicks given lactose. The organisms were susceptible to penicillin and their appearance was coincident with poor growth. The same authors also detected *Aerobacter aerogenes* in the caeca of chicks given penicillin. Elam, Jacobs, Tidwell, Gee and Couch (8) observed that penicillin greatly reduced the number of clostridia present in the intestinal tract of the chick. Later, Elam, Jacobs, Fowler and Couch (9) estimated the clostridial population in the faeces of birds in old and new premises. They found that penicillin given by mouth stimulated growth and reduced the faecal clostridia in old quarters; in new premises, where no growth stimulation by antibiotics was observed, the faecal clostridial count was lower and was unaffected by penicillin in the diet.

In species other than poultry there is also evidence of an environmental influence on growth response to antibiotics. Bartley, Fountaine, Atkeson and Fryer (10) observed a marked improvement in growth rate and a reduction in the incidence of minor diseases when aureomycin was given to calves reared in a barn where previously colds and scours had been prevalent. In a later experiment (11) the effect of aureomycin on calves reared under more favourable conditions in a new barn was studied. On this occasion the control calves grew as well as those that had received aureomycin in the previous experiment. Unlike our experience with chicks, however, in the new quarters there was still a significant response to aureomycin in both gain in weight and reduction in the incidence of disease, although to a lesser extent than in the old premises. As the authors point out, experiments with calves are usually of longer duration than those with chicks and it is not unlikely that the barn could have become contaminated with undesirable organisms before completion of their work. Investigation at this Institute (unpublished) has shown that the growth of home-bred heifer calves born and reared on the premises was not improved by aureomycin or penicillin, whereas calves brought in from other farms, and reared therefore in a foreign environment, grew significantly better if given aureomycin or penicillin.

Although in general the growth of pigs is improved by the addition of antibiotics to the diet, there have been reports (12, 13) of failure to obtain any marked response. Speer *et al.* (12) suggest that in premises where the 'disease level' (14) is low, little or no benefit is to be expected from antibiotics. This suggestion is borne out by the experience of Whitehair (15) who showed that baby pigs delivered aseptically by caesarean section and reared in isolation did not respond to aureomycin. Williams (16) showed that in his first experiment in newly constructed animal quarters baby pigs reared on a synthetic milk did not respond to supplements of aureomycin, but in the second and subsequent experiments growth of the pigs without aureomycin became less good. A summary by Braude (17) of experiments on the use of antibiotics in pig feeding in the United

Kingdom showed that where the general level of growth was poorest the greatest improvement resulted from antibiotics.

Although it is apparent that environment affects the occurrence and magnitude of growth stimulation by antibiotics, it is not clear which particular factor or factors in the environment are responsible. Since antibiotics are potent bacteriostatic agents it is likely that in the presence of frank disease some improvement can be expected, simply as a result of therapeutic action. It is probable that the findings of Bartley *et al.* (10, 11) with calves in new and old rearing-barns can be thus explained. The experience of Roy, Shillam, Palmer and Ingram (18) with colostrum-deprived calves was most likely a demonstration of the therapeutic effect of aureomycin, particularly in view of the relatively high dose (250 mg./day) that was given. These workers obtained significantly greater live-weight gains, a reduction in scouring and some indication of a lowered mortality in calves deprived of colostrum but given aureomycin in the milk, and they pointed an analogy between the antibacterial value of colostrum and of the antibiotic. Similarly with pigs, since the early report of Carpenter (19) there is considerable evidence that unthrifty animals benefit from antibiotics in the diet. These results, as well as those summarized by Braude (17), may also reflect a reduction in the incidence of minor ailments by the use of antibiotics in the food.

With poultry, however, and to a lesser extent with pigs, a benefit is frequently obtained from the feeding of antibiotics in the absence of any recognizable disease. This phenomenon could be explained in terms of the effect of antibiotics on the types or numbers of the bacterial population. Thus the poorer growth obtained in long-used, 'stale' premises may be the result of a pathological condition so slight as to pass unrecognized, or an abnormally high bacterial population which, though consisting of non-pathogens, may by sheer weight of numbers be detrimental to the young growing animal. In eitherevent, the antibiotic might be expected to counteract the adverse effect either by eliminating the agent responsible for the subclinical condition or by reducing the total bacterial population. Despite much work the evidence on this second possibility is still by no means clear, moreover our own findings (5) accord more nearly with the first alternative, and suggest that the condition may be due to a living agent inhabiting the gut of birds not receiving penicillin. The findings of Cooper and Gordon (6) that a growth-depressing condition counteracted by penicillin could be transmitted in a crude lung filtrate also support this view, but, although their premises were originally 'infected' with gut contents from our chicks, there is no evidence to determine whether the condition transmitted in the lung filtrate was identical with that induced by the gut contents. In considering the nature of the growth-depressing condition in 'stale' premises, the possibility cannot be overlooked that a change in bacterial metabolism, rather than in types or numbers, is responsible. Such a phenomenon would not be easily disclosed by ordinary bacteriological techniques, but its existence might explain the striking lack of agreement between the results of different groups of workers who have tried to identify a specific organism concerned in growth stimulation by antibiotics. For instance Smyser, Cleverdon, Kulp and Matterson (20), in contrast to Elam *et al.* (9), reached the conclusion that *Cl. perfringens* has no significant role in stimulation of chick growth by dietary antibiotics. It is possible that even if submaximal growth in the absence of antibiotics is the result of a specific agent this may not be the same in all environments.

In contrast to the widely held idea that antibiotics may eradicate organisms detrimental to growth, the possibility also exists that antibiotics may encourage the development of an internal or external microflora of benefit to animal growth. Once again, different environments may favour the establishment of different organisms in the gut and hence, directly or indirectly, influence the nutrition of the host. The report by Romoser *et al.* (7) of the appearance of *Aer. aerogenes* in the caeca of chicks given penicillin was followed by further experiments (21) in which the feeding of a viable culture of *Aer. aerogenes* to chicks resulted in a growth response that partly replaced the effect of antibiotics. In parallel experiments Anderson and his colleagues gave live cultures of certain strains of *Escherichia coli* to chicks (22) and poults (23) and obtained weight increases in the absence of penicillin. These authors state that no organisms of the genus *Aerobacter* have been encountered in the caeca of chickens in their laboratory. Thus, although the two groups of workers differed in detail both were able to associate distinct bacterial 'milieux' with improved growth. It is conceivable that optimal growth in animals requires a certain internal bacteriological balance, but that this is not necessarily the same in all environments or for all species. Antibiotics in the diet may assist the establishment of the critical flora, but in environments where the appropriate organisms already flourish their use is redundant.

The findings of Roine, Ettala, Raitio and Vartiovaara (24) are of interest in this connexion, although they deal with an adverse effect of antibiotics. These authors found that aureomycin was fatal to guinea-pigs when given in the diet at levels which normally promote growth in other species. The detrimental effect of aureomycin was accompanied by marked changes in the caecal flora, and in particular the numbers of *Listeria* organisms were considerably increased. *Listeria monocytogenes* is a known parasite of some domestic animals and produces a syndrome similar to that observed in the aureomycin-treated guinea-pigs. It is apparently possible, therefore, for listeria to become predominant without the aid of aureomycin. These results are an example of an antibiotic promoting the dominance of a certain flora, albeit in this instance a detrimental one.

It has been amply shown that antibiotics given in the diet decrease the weight of the gut (25, 26, 27, 28). Our experiments (27) showed that the decrease in weight was the result of a thinning rather than a shortening of the gut. We also observed (27), although the scope of these particular experiments was small, that no effect on the gut was apparent in premises where no growth response to penicillin occurred. From these results it might be postulated that the thicker gut in untreated animals is a reaction to the establishment of an unfavourable

flora; that the noxious agent or agents are susceptible to antibiotics and are absent from environments where no growth response and no effect on the gut are observed. Furthermore, the thickening of the gut wall could result in less efficient absorption of nutrients, which would account for our finding (29) that vitamin A reserves are higher in the livers of birds in environments where there is no growth response to antibiotics. Such a hypothesis might also be extended to other vitamins and essential nutrients.

The foregoing discussion has been based upon the assumption that antibiotics bring about their effect on growth through an action on micro-organisms, but there are some observations with which such an assumption is not compatible. Elam, Gee and Couch (30) found that penicillin, made inactive for test organisms, by autoclaving promoted growth when injected into chicks. Later Fell and Stephenson (31) obtained a growth response in chicks to penicillamine, which supports the suggestion tentatively put forward by Elam *et al.* (30) that the antibiotic molecule, or a fragment of it, might act as a metabolite within the body of the bird. More recently Taylor and Gordon (32) showed that penicillin inactivated by any of three different methods retained a considerable proportion of its growth-promoting action for pigs; no penicillin could be detected in the gut, serum or urine of the experimental animals. These authors suggest that penicillamine may be responsible for the growth effect, being the only stable end-product common to all the inactivation procedures.

Perhaps the most striking evidence for a non-bacterial mechanism of action is the recent work of Luckey, Gordon, Wagner and Reyniers (33), who obtained significant growth responses to antibiotics in germ-free chicks and poults. In earlier experiments Luckey (34) had found a consistent small decrease in the growth rate of germ-free chicks given succinyl-sulphthiazole or antibiotics in the diet. These results were in accordance with those of Jukes and Williams (35) who failed to observe growth stimulation in sterile chick embryos injected with antibiotics. However, Luckey and his colleagues interpreted the lack of response in their germ-free chicks as a possible toxic effect of too high a dosage, and smaller doses were therefore used in the later work. With these lower levels of antibiotics, small but significant growth increases occurred which the authors claim to « allow the presumption that antibiotics did stimulate growth in these germ-free birds ». As no living micro-organisms could be detected in the birds by accepted visual and cultural techniques it must be concluded that on this occasion at least the antibiotics produced their effect by direct action on the birds receiving them, rather than by virtue of their anti-bacterial properties. Analogous experiments on plant growth have been done by Nickell and Finlay (36), who found that several antibiotics at low dose levels stimulated the growth of duckweed plants cultured in aseptic conditions.

Thus, although there is overwhelming evidence that the growth-promoting effect of antibiotics depends to a large extent on their antibacterial properties, the importance of the work in aseptic environments cannot be overlooked. It is generally accepted that antibiotics have a direct effect on certain metabolic processes, hence it is conceivable that, given in the diet, they exert a small but definite pharmacological action which contributes to their beneficial effect on growth, but that in ordinary environmental conditions such an action may pass unnoticed because of the overriding predominance of the bacteriological effects.

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# Effect of antibiotic feeding on the intestinal microflora of animals

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(*Received* 4 *April* 1955)

Pasteur (1) in 1885 was perhaps the first worker to suggest that the microorganisms of the intestinal tract are important to the health of the host animal. Osborne and Mendel in 1911 (2) presented evidence which suggested vitamin synthesis by intestinal microorganisms of the rat, and during the next two decades numerous research reports were written on the ability of coprophagous rats to subsist on diets which were deficient in one or more vitamins. Later the study was extended to include still other experimental animals than the white rat, and more precise studies involving vitamin assays and population studies were instituted. The work of Mitchell and Isbell (3) was noteworthy in that they demonstrated the extent to which B-vitamins were synthesized in the cecum of the rat and also showed the sites of absorption of dietary and synthesized vitamins. At nearly the same time Marshall *et al.* (4) contributed to the understanding of the role of intestinal bacteria in animal nutrition when he reduced the numbers of intestinal bacteria and simultaneously produced vitamin deficiencies in rats by feeding sulfaguanidine. This type of vitamin deficiency was studied extensively, most notably by researchers at the University of Wisconsin (5-10), and it was established that the coliform group of intestinal organisms was consistantly reduced by sulfonamide feeding. The accompanying vitamin deficiencies could be corrected by addition of various extractives from which more and more of the presently known B-vitamins were isolated.

Still another approach to the problem of determining the importance of intestinal microorganisms in nutrition of the host was the application of germ-free methods to animals. This technique was initiated by Nuttall and Thierfelder in 1895 (11) and was refined and expanded by Reyniers and co-workers (12) at the Lobund Institute. These studies indicate that various small animals kept under apparently aseptic conditions do not grow as well as controls which harbor intestinal microorganisms, even when rations are supplemented with all known vitamins and with extractives to supply unidentified growth factors.

With the advent of antibiotics a new chapter in the study of the nutritional role of intestinal flora was opened. The early work on the effect of antibiotics in animal nutrition was reviewed by Stokstad (13), and by MacKimpson (14). Opinion as to the mechanism of action of antibiotics in causing growth promotion in animals was divided. Three different views, with many research results to support each, have been taken :

(*a*) Antibiotics eliminate bacteria which produce harmful substances ('disease level' theory).

(*b*) Antibiotics eliminate intestinal bacteria which absorb or destroy nutrilites needed by the host.

(*c*) Antibiotics enhance the growth of intestinal microorganisms which synthesize nutrilites required by the host.

### *In support of the first theory*

The report by Luckey *et al.* (15) that antibiotics do not stimulate the growth of germ-free animals was a convincing argument, and the demonstration by Spear *et al.* (16) that healthy, well nourished weanling pigs did not respond to aureomycin supplementation when fed an 18 % corn and soybean supplemented ration and reared in isolated, carefully cleaned, and disinfected pens with concrete floors which had not been previously used for pigs, was another of the early findings which has been confirmed with other antibiotics and other animals by a number of other researchers (17, 18, 19). Many bacteriological studies have shown the depression of one or more types of intestinal microflora by dietary antibiotics. Sieburth *et al.* (21), Larson and Carpenter (20) and Williams and co-workers (22) all reported that fecal clostrida were decreased in numbers when antibiotics were fed to chicks. The importance of enterotoxin forming clostridia was questioned how-

ever, when Williams *et al.* (23) showed that antitoxins for enterotoxins did not cause growth effects in chicks. Moore *et al.*(10) noted that feeding streptomycin decreased the coliform content of the ceca of chicks. Guzman-Garcia *et al.* (24) noted that penicillin caused a decrease in the anaerobic count in the cecum and intestine of the rat during the first week on experiment.

### *In support of the second theory*

March and Biely (25) indicated that the first group of bacteria to be affected by aureomycin was the lactobacilli which they regard as competitive with the host for some of the dietary vitamins. Anderson, Slinger and Pepper (26), Johansson and Sarles (9) reported that antibiotics caused a reduction in the numbers of lactobacilli in the cecum of the chick. Anderson *et al.* (27) reported that penicillin decreased the number of enterococci in the cecum of the chick.

### *In support of the third theory*

The number of papers which shows that traces of antibiotics in the ration cause an increase in certain types of intestinal organisms is so great as to be difficult to be handled easily. Moore (10) reported that streptomycin increased lactobacilli and yeasts, and Cunningham and Slinger (27) found that penicillin increased coliforms other than *Escherichia coli*, in the cecum of the chick.

Romoser, Shorb and Combs (28) and Anderson, Slinger and Pepper (26) isolated coliforms from chicks on aureomycin and penicillin supplemented rations and found that feeding these cultures to chicks caused slight

TABLE I.

*Estimated number of microorganisms per gram of wet feces (values × 10⁶)*

| Organisms | Lot No(*) | Weeks on experiment (**) 0 | 1 | 2 | 3 |
|---|---|---|---|---|---|
| Coliforms | I | 2 | 3 | 135 | 31 |
| | II | 2 | 4 | 25 | 103 |
| Fungi | I | 0.57 | 5 | 5 | 2 |
| | II | 0.89 | 2 | 4 | 266 |
| Anaerobes | I | —— | 1155 | 2740 | 1086 |
| | II | —— | 2900 | 1218 | 158 |
| Enterococci | I | —— | 3000 | 9 | 3 |
| | II | —— | 3260 | 3 | 22 |
| Glucose fermenters | I | —— | 2 | —— | 10 |
| | II | —— | 71 | —— | 255 |
| Proteolytics | I | —— | 0.03 | —— | 2 |
| | II | —— | 0.20 | —— | 83 |
| Total count (aerobic) | I | 1220 | —— | 1043 | 1937 |
| | II | 728 | —— | 1722 | 2394 |

(*) Lot I. — Basal ration.
Lot II. — Basal ration + 5 mg. aureomycin HCl per lb. ration.

(**) Samples collected from the following number of pigs : 0 wk., 3; 1 wk., 2; 2 wks., 2 and 3 wks., 1.

but consistent growth responses in the presence or absence of antibiotic.

Quinn, Lane, Ashton, Maddock and Catron (29) in a study of the distribution of types of intestinal organisms of swine receiving aureomycin compared with control animals not receiving aureomycin obtained the results presented in table I.

It will be noted that enterococci are the only organisms which decreased in population density during the period of observation. Also, it should be noted that this group tended to disappear from the intestinal tract whether or not aureomycin was present in the ration of the pigs.

The total counts of aerobic organisms in the feces from fastgrowing swine frequently included a high percentage of *Bacillus* spp. Since Lewis *et al.* (30) had reported favorable results from animal feeding trials with such organisms, the most frequently occurring type, *Bacillus subtilis panis*, was included in swine feeding trials (see Table II).

TABLE II. — *Summary of averages*

| Treatment (*) | Ave. daily gain | Ave. daily feed | Feed per cwt. gain |
|---|---|---|---|
| Basal | 1.24 | 3.29 | 265 |
| Basal + Aureomycin | 1.29 | 3.28 | 255 |
| Basal + *A. niger* | 1.15 | 2.92 | 252 |
| Basal + *A. chevalieri* | 1.20 | 3.17 | 265 |
| Basal + *A. flavus* | 1.40 | 3.55 | 253 |
| Basal + *Bacillus subtilis panis* | 1.28 | 3.00 | 234 |

(*) 4 pigs per treatment.

In general, the intestinal organisms plated out from the feces of animals which received aureomycin in the ration were slower growing than were the microorganisms recovered from feces of control animals which did not receive aureomycin. Fungi showed the reverse, however, as the presence of antibiotics in the plating medium seemed to increase the rate of colonial growth.

Since it was observed earlier that swine which were fed in wirebottomed crates did not show significant growth stimulation from the feeding of aureomycin Quinn, Story, Catron, Jensen and Whalen (31), it was felt that aureomycin was increasing intestinal synthesis of a growth factor which was absorbed in effective amounts only when refection occurred. In a search for the organisms responsible for synthesis of the growth factor, Quinn, McKimpson, Underkofler, Ashton, and Catron (32) found that fungi were increased more, percentagewise, than other groups of intestinal microorganisms studied, when aureomycin was used to stimulate the rate of growth of swine. The result of feeding some of these fungi to swine is given in Table II.

A 10 % level of supplementation with whole fungus cultures was selected on the basis of rat response to various levels from 1.25 % to 40 % fungus culture in the ration. The culture medium for the fungi and bacillus was Czapek-Dox medium plus 2 % sucrose and 0.1 % corn steep liquor. The gain of each pig in the basal plus *Aspergillus flavus* 18 feeding lot was consist-

ently higher than its replicate in the basal lot. The increase of 0.16 pounds in average daily gain in the *A. flavus* 18 fed swine was significant at $P = 0.06$ over basal. The feeding of *Bacillus subtilis* morphotype *panis* promoted a saving of 34 pounds of feed per 100 pounds of gain, which is also significant over basal at $P = 0.06$.

At this point a search was begun for a microbiological assay for the *A. flavus* 18 factor. Since it was felt that the presence of this factor in the intestinal tract of well-doing swine would also encourage the growth of dependant microorganisms fecal samples were serially diluted and the resulting colonies on agar that contained the *A. flavus* 18 culture filtrate were screened for auxotrophs requiring this factor, using the method of Adelberg and Myers (33). The minimal medium used in picking auxotrophs contained the known vitamins plus certain crude supplements such as peptone, yeast extract and corn steep liquor.

The vitamins are present in this medium at the same concentration as in the corn-soy-mineral basal ration. Vitamins were sterilized by sintered glass filtration, and were added aseptically to the rest of the heat sterilized medium, including the heat-stable *A. flavus* 18 filtrate factor.

Although auxotrophs belonging to *Micrococcus*, *Lactobacillus* and *Streptococcus* were isolated, the best microorganism for turbidimetric assay proved to be the auxotroph designated as *Pediococcus* strain AF. This organism failed to grow on the above minimal medium even after one week of incubation, but growth was rapid in minimal medium plus 10 % *A. flavus* 18 filtrate. Unless care is exercised in maintaining a dependant strain, this organism will acquire the ability to grow slowly in the minimal medium.

When added at 20 % level to tryptone-glucose-beef extract-yeast extract broth, the *A. flavus* 18 filtrate caused marked increase in the rate of growth of various wild strains of organisms such as *Lactobacillus casei*, *Saccharomyces cerevisiae* and *Streptococcus lactis*, while the effect on some thirty other stock cultures was less marked.

Balloun (34) found that 20 % of *A. flavus* 18 filtrate in chick rations caused an average weight increase over basal of 28 grams at three weeks compared to 26 grams for chicks receiving 5 mg. of aureomycin per pound of ration.

Strong alkali and hot concentrated acid inactivate the *A. flavus* filtrate factor, while heavy metals precipitate it from aqueous solution. Some purification of the factor was carried out by dissolving condensed filtrate, in 50 % aqueous methanol, followed by precipitation of the active fraction into the aqueous phase by addition of benzene. When this material which was soluble in water and in aqueous alcohol was assayed microbiologically, it proved to have approximately twenty times the growth promoting activity of the starling *A. flavus* 18 culture filtrate. Balloun (34) added this purified material to chick rations at a level corresponding to 10 % *A. flavus* 18 culture filtrate and obtained a 92 g. average gain per bird at two weeks compared to 82.5 g. average gain on basal ration. This difference was statistically significant at $P = 0.01$.

Diaz (35) fed a concentrate of the *A. flavus* 18 filtrate to weanling pigs and obtained the results shown in table III.

TABLE III

*Response of weanling pigs to supplementation with* A. flavus 18 *filtrate*

| Ration treatment: No. | Ration treatment: Additions to basal | No. Pigs | Days on expt. | Initial weight (*) | Final weight (*) | Total gain (*) | Av. daily gain (*) | Feed per lb. gain (*) |
|---|---|---|---|---|---|---|---|---|
| 1. Control | None | 6 | 72 | 18.5 | 99.8 | 81.3 | 1.13 | 2.65 |
| 2. Antibiotic | 5 mg. aureomycin per lb. of feed | 6 | 63 | 18.8 | 106.7 | 87.9 | 1.39 | 2.58 |
| 3. *A. flavus* 18 filtrate | 10 % level equivalent | 6 | 63 | 18.7 | 107.0 | 88.3 | 1.40 | 2.54 |

(*) In pounds.

It will be noted that both aureomycin and *A. flavus* 18 filtrate gave definite, and almost identical, growth responses under these conditions.

At present, the *A. flavus* 18 factor has been concentrated approximately 200 times by fractional precipitation with salt and methanol from saturated aqueous solutions. This highly active material is under test with rats and weanling pigs.

## DISCUSSION

The evidence presented seems to indicate that the manner in which antibiotics act in stimulating the growth of animals is very complex. Certainly the intestinal flora is altered both qualitatively and quantitatively, and the results of antibiotic feeding depend on the entire environmental, nutritional, genetic, and microbial picture existing at the time of the experiment.

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# Etude de quelques propriétés biochimiques de la flore intestinale du porc. Influence des antibiotiques

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(*Reçu le 28 juillet 1955*)

La vitesse de croissance des animaux peut être stimulée par l'ingestion de petites quantités de diverses substances ; on sait que certains antibiotiques possèdent notamment cette propriété. Toutefois, les mécanismes de cette action sont mal connus. L'hypothèse d'une influence de ces substances sur la flore digestive a été souvent avancée (1, 2). Les recherches sur la densité et la nature de la population microbienne des contenus digestifs et des fèces n'ont pas apporté d'éclaircissements substantiels.

Dans le présent travail, les modifications de certaines propriétés biochimiques de la flore intestinale du porc sous l'influence des antibiotiques ont été particulièrement étudiées. La validité des résultats obtenus au moyen de techniques *in vitro* a été vérifiée *in vivo*, par l'étude de l'absorption de certaines substances au niveau intestinal.

Nous avons en effet mis en évidence les points suivants :

(*a*) Action sur le métabolisme azoté : influence des antibiotiques sur les désaminases microbiennes ; identité d'action de certains antibiotiques et de diverses substances (notamment $Cu^{++}$ et tannin), sur les désaminases microbiennes ; influence des antibiotiques sur l'amination directe des acides cétoniques ; influence sur les transaminations.

(*b*) Action d'épargne au niveau intestinal, sur certains métabolites tels que la choline.

(*c*) Modification de l'absorption intestinale de certains métabolites en présence d'antibiotiques.

(*d*) Modifications de la flore intestinale (densité microbienne, vitesse d'autolyse).

Parmi les antibiotiques étudiés, l'auréomycine exerce le plus souvent une action très marquée sur les différents phénomènes étudiés précédemment. Pour cette raison, cet antibiotique a été utilisé comme corps de référence.

## TECHNIQUE EXPÉRIMENTALE

Le contenu untestinal du porc est prélevé dès l'abattage, à différents niveaux (intestin grêle, caecum, côlon). La flore est isolée par centrifugation fractionnée (3).

La suspension microbienne, amenée à une concentration déterminée, sert à ensemencer le substrat à étudier. On opère soit en culture proliférante, soit en phase stationnaire. Les conditions de milieu (pH, température, densité microbienne et concentration des divers constituants) sont aussi proches que possible de celles existant dans le milieu intestinal.

Dans tous les cas, on étudie la cinétique de la réaction dans des intervalles de temps compatibles avec ceux de la digestion normale.

Le dosage de l'azote total et de l'azote ammoniacal est effectué par la méthode de micro-diffusion de Conway (4). Les acides aminés sont dosés par chromatographie quantitative sur papier. Les acides cétoniques sont isolés à l'état de dinitrophénylhydrazones et dosés colorimétriquement.

## RÉSULTATS

### *Métabolisme azoté*

*Action sur les désaminases microbiennes.* — La flore complexe de l'intestin du porc cultivée sur bouillon viande-foie, provoque la libération d'ammoniac dans le milieu de culture. L'autolyse des corps microbiens n'est responsable que pour une très faible part de cette libération. Nous avons vérifié que l'ammoniac ne se forme pas non plus par une réaction de Stickland. Son origine réside dans la désamination directe des acides aminés ; il est aisé de le démontrer par l'étude des cultures stationnaires en présence de substrat pur. On trouvera, dans le tableau I, les taux de désamination de divers acides aminés par la flore intestinale du porc après 40 heures d'incubation.

TABLEAU I

*Pourcentage de désamination de divers acides aminés*

| Acide aminé | % |
|---|---|
| Acide glutamique | 74.8 |
| Acide aspartique | 89.5 |
| Arginine | 70.0 |
| Citrulline | 83.0 |
| Ornithine | 1.8 |
| Histidine | 78.5 |
| Lysine | 10.5 |

Flore du caecum du porc ; 40 heures d'incubation.

Certains antibiotiques, et en particulier l'auréomycine et la terramycine, inhibent ces désaminations. En revanche, certains autres tels le chloramphénicol, n'exercent qu'une action négligeable (tableau II).

Il ne semble pas que la propriété d'inhiber les désaminases soit liée au pouvoir antibiotique ; en effet, d'autres substances qui présentent des propriétés inhibitrices très nettes, sont sans action sur la croissance microbienne (tableau III). Le cuivre et le tannin, ainsi que l'acide 3-nitrophénylarsinique, présentent notamment cette propriété.

*Action sur les aminations.* — La flore intestinale du porc, en présence d'ammoniac et d'un acide cétonique, synthétise l'aminoacide correspondant. Cette réaction est limitée par l'action des décarboxylases microbiennes sur le céto-acide initial et par la dégradation de l'aminoacide formé.

TABLEAU II

*Influence de divers antibiotiques sur le taux de désamination de l'arginine*

| Antibiotique | % |
|---|---|
| Auréomycine | 21.2 |
| Terramycine | 20.4 |
| Pénicilline | 57.0 |
| Bacitracine | 66.1 |
| Streptomycine | 64.0 |
| Chloramphenicol | 96.6 |
| Nisine | 80.0 |

Les résultats sont exprimés en % de l'activité du témoin. Les antibiotiques sont utilisés à la concentration de 10 µg./ml.

TABLEAU III

*Influence de diverses substances sur le taux de désamination de l'arginine*

| Substance | Concentration | % (*) |
|---|---|---|
| $CuSO_4$. | 250 µg./ml. | 33.6 |
| | 25 µg./ml. | 76.8 |
| Tannin. | 500 µg./ml. | 11.4 |
| $HgCN_2$ | $10^{-3}$ M | 1.4 |
| | $10^{-4}$ M | 4.5 |
| | $10^{-5}$ M | 100 |
| Acide 3-nitro-4-hydroxyphényl-arsinique. | 100 µg./ml. | 45 |
| | 10 µg./ml. | 94 |
| 2,4-Dinitrophénol α. | 25 µg./ml. | 12.5 |
| | 2 µg./ml. | 78 |

(*) Les résultats sont exprimés en % de l'activité du témoin.

Le taux de fixation de l'ammoniac dans l'essai témoin (40 % en moyenne) présente un maximum après environ 16 heures d'incubation. En présence de l'acide pyruvique, l'ammoniac disparu se retrouve alors quantitativement, sous forme d'alanine, qui est ensuite rapidement catabolisée. Après 40 heures d'incubation, il ne reste plus d'acides aminés, et le taux de fixation de l'ammoniac est abaissé (20 %). L'auréomycine ralentit à la fois la vitesse de fixation de l'ammoniac et la vitesse de désamination de l'acide aminé formé. Il en résulte que le taux final de fixation est plus élevé en présence d'antibiotique ; il est de 30 % environ et l'on retrouve dans le milieu une quantité notable d'acides aminés : alanine, acide aspartique, acide glutamique, acide γ aminobutyrique.

*Action sur les transaminations.* — L'asparagine est rapidement dégradée par la flore intestinale du porc. La liaison amide est d'abord hydrolysée avec formation

d'ammoniac et d'acide aspartique, lequel est désaminé à son tour. En présence d'un céto-acide, la réaction est plus rapide (5). Dans ce cas également, l'ammoniac libéré se fixe sur l'acide cétonique.

Nous avons observé que l'auréomycine ralentit fortement la réaction, par blocage de la désamination du groupement amide. Comme dans le cas précédent, l'antibiotique a exercé un rôle d'épargne envers la fonction azotée.

*Action d'épargne directe sur certains principes*

La flore intestinale du porc dégrade rapidement *in vitro* la choline, en produisant de la triméthylamine. Diverses substances, parmi lesquelles l'auréomycine se place au premier rang, inhibent cette destruction (tableau IV).

TABLEAU IV

*Dégradation de la choline par la flore du cæcum du porc*

| Inhibiteur utilisé | Concentration | % (*) | |
|---|---|---|---|
| | | Après 16 h. | Après 40 h. |
| Auréomycine. | 15 µg./ml. | 0 | 0 |
| | 10 µg./ml. | 2 | 1.9 |
| | 1 µg./ml. | 25 | 85 |
| Pénicilline G. | 10 µg./ml. | 8.5 | 83 |
| Chloramphénicol. | 10 µg./ml. | 89.5 | 100.5 |
| Terramycine. | 10 µg./ml. | 0 | 31.6 |
| | 1 µg./ml. | 45.6 | 81.6 |
| Tannin. | 500 µg./ml. | 0 | 78 |

(*) Les résultats sont exprimés en % du témoin.

*Taux d'ammoniac dans le sang prélevé dans la veine porte*

Des dosages successifs dans le sang d'animaux porteurs d'une canule de la veine porte ont montré un abaissement significatif du taux d'ammoniac lorsque les animaux ont ingéré de l'auréomycine.

Les courbes de libération de l'ammoniac au cours du dosage par la méthode de Conway (4) sont atypiques lorsque l'animal a ingéré l'antibiotique. Ce fait permet de supposer que l'action de l'auréomycine s'exerce également au niveau sanguin.

*Action sur la flore*

La croissance bactérienne *in vitro* en présence d'auréomycine, présente les caractéristiques suivantes, par rapport au témoin : (*a*) augmentation de la période de latence, (*b*) accroissement de la densité microbienne limite, (*c*) accroissement de la vitesse d'autolyse.

En accord avec ces observations, nous avons observé *in vivo* une diminution du nombre de micro-organismes dans l'intestin grêle et un accroissement dans le cœcum et le colon. La teneur des corps microbiens en azote était également plus faible.

## INTERPRÉTATION ET DISCUSSION

Parmi les différentes actions biochimiques dues à la flore intestinale du porc et susceptibles d'être modifiées par les antibiotiques, les désaminations présentent une importance particulière. En effet, Michel et François (6) ont mis en évidence la relation existant, pour différents antibiotiques, entre l'indice de croissance et l'aptitude de l'antibiotique à inhiber la désamination de l'arginine.

Par ailleurs, Barber *et al.* (7) ont montré récemment, que le sulfate de cuivre à la dose (exprimée en $Cu^{++}$) de 250 mg./kg. de ration stimulait la croissance du porc. Or, nos résultats montrent que l'ion $Cu^{++}$, aux doses indiquées ci-dessus, est un puissant inhibiteur des désaminations. Par ailleurs, l'acide 3-nitro-4-phénylarsinique, qui exerce une action sur la croissance, possède également la propriété d'inhiber les désaminases de la flore intestinale du porc. La diminution du taux d'azote ammoniacal du sang prélevé dans la veine porte lorsque l'animal ingère de l'auréomycine, apporte une confirmation directe *in vivo* à l'inhibition des désaminases de la flore intestinale.

Nous vérifierons ultérieurement si certaines substances capables d'inhiber les désaminations sont également capables de stimuler la vitesse de croissance. Le tannin et le 2,4-dinitrophénol, rentrent notamment dans cette catégorie de substances.

Nous ne pouvons indiquer actuellement si la modification de propriété biochimique affecte l'ensemble de la flore initiale ou bien si l'équilibre de celle-ci est modifié par l'antibiotique.

Les modifications des propriétés biochimiques de la flore présentent certainement une importance considérable, et récemment, Melnykowycz et Johansson (8) ont montré que la décarboxylation des acides aminés par le contenu intestinal du rat était également inhibée par la chlorotétracycline. De même, Larson et Hill (9) ont mis en évidence une diminution de l'activité métabolique des bactéries des animaux recevant de l'auréomycine.

L'action d'épargne directe que nous avons mise en évidence avec la choline, peut vraisemblablement s'étendre à d'autres substances : vitamine $B_1$, $B_2$, etc. Elle présente sans aucun doute une signification nutritionnelle importante.

Enfin, la modification de la cinétique de la libération d'ammoniac du sang, lorsque l'animal a ingéré de l'auréomycine, permet de penser que l'antibiotique modifie également certaines propriétés biochimiques de ce milieu.

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# Germfree life methodology (gnotobiotics*) and experimental nutrition (**)

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(*Received* 5 *July* 1955)

It is possible to approach nutrition theoretically and consider the basic elements involved (1). Broadly speaking, these are the diet and the host system which utilize it. If these factors are accepted together with the environment as part of an experimental equation, it is obvious that the degree of purity of each must be taken into account together with the possible degree of standardization of each. As in all experimental science, the ultimate aim is exact control of variables and where the possibility of control is less, at least it should be possible to account for the degree of variation and determine the parameters. Unless the units involved in an experiment can be satisfactorily identified and isolated to the necessary degree of purity, the exactness of the results will always be in question. Therefore, in defining the host and the diet, it is necessary to first isolate each from the complex in which they ordinarily exist and to keep them isolated until they can be brought together as pure units.

Now the host system, if it is an animal, does not exist in nature as a pure unit. Rather it is a combination of biological systems, animal, microbial, etc. Likewise, the natural diet may be a composite of animal and vegetable matter difficult to define exactly. In this respect the history of nutrition has been marked by a gradual breakdown of the crude, poorly defined diets into better chemically defined components. The future probably holds a still further purification to ultimate units in an effort to define metabolic pathways and a closer study of nutritional interrelationships.

The animal on the other hand has not been so critically evaluated as a component part of the experimental equation. A logical first step in its purification would be to separate it from the contaminants with which it has always been associated from birth.

If we are to observe the basic principles of experimentation, it is necessary to make more certain that the animal and the diet meet the criteria of purity, *i.e.*, that the diet be defined chemically and the animal be a pure host system, rather than a combination of host systems as is the case when it is contaminated. The same holds for the environment.

An example in point, is the science of bacteriology which rests on a foundation of the pure culture concept. The microbe is isolated from the complex in which it naturally exists and is grown as a pure culture. The diet or nutrient may be more or less chemically defined. The methodology employed permits the microbe to be studied as a pure culture, or to be brought into contact with other pure cultures. When the animal is considered as a part of an experimental system, then logic demands that the pure culture concept be extended to the host and that this host be grown in the absence of contaminants. That this has not been the case is an accident of time since the demands of the past were satisfied with a contaminated animal system. However, in the future, as new frontiers are approached and the demands of the experimental method become more exacting, the situation must be corrected.

What I propose here is to extend the pure culture concept to nutrition through the study and use of germfree animals. Indeed, it is this thesis of extending the pure culture concept to experimental biology and medicine which prompted my studies in 1928 and has constituted the framework of the research of my associates and myself since.

At the present state of knowledge a germfree animal may be defined as a pure animal system free from all detectable contaminants. The term contaminant refers to life other than that produced by the host cells. Thus contaminants may come from the external environment of the host or they may be carried from host to host through successive generations and considered part of the internal environment. It is obvious that the detection of such contaminants will always be a measure of the techniques applied and this, in turn, depends on how far the techniques used have progressed toward a recognized demand. As the matter now stands the

(*) The methodology which has grown up around germfree life research has with time made it obvious that special terminology is needed (2). It is with this in mind that one of the Lobund group (P. C. Trexler) has suggested the term 'gnotobiotics' (Gr. γνοσις = known; βιος = life) to designate the field as well as the statuts of an animal. The term includes the 'germfree' animal reared in a sterile environment in the absence of all known contaminants as well as similar animals living in association with only known microbial associates. Under these biologically defined conditions, the animal becomes a 'gnotobiote', the environment a 'gnotobiotope', the process is 'gnotobiotization', (verb: to 'gnotobiotize') and the animal becomes 'gnotobiotic'. The state of freedom from unwanted or unknown contamination is 'gnotobiosis'.

(**) These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Notre Dame, NR 131-067, and by U. S. Public Health Service Grant nr. A-566.

techniques of microbiology, physiology, pathology, serology, etc., may be incorporated into the scheme of detection. Probably the best indicator of contamination is the germfree animal and its environment because they provide an optimum for growth of contaminants and the animal is an excellent sampling device. So, in short, at the present time a germfree animal is one which is free from all living microbes, parasites and detectable viruses and lives in an environment likewise free from these. The future may reveal that contaminants in the host system exist beyond the techniques presently available for detecting them, *e.g.*, symbiotic viruses, and new definitions, new methodology and new techniques may be necessary. However, this is a stepwise progression toward greater purity of the host and the result of increased theoretical demands. Research in this direction cannot progress in the presence of recognizable contaminants but only when each level of existence is surveyed with adequate methodology.

## HISTORICAL (*)

The merit of having performed the first germfree experiment is unquestionably that of Duclaux working with peas and beans (3). As far as the original idea and theoretical implications are concerned, Pasteur's name is usually mentioned. In this respect we may be a little unjust to Duclaux seeing in him a « pair of hands ». The latter was certainly not the case as is quite evident from the way he elaborated his thesis. He also must have had a wider background, branching into animal physiology. Pasteur (4) mentions his distinguished contributions which we have so far been unable to locate. Whatever the proper credit distribution might be, the thought behind this experiment ramified at least in two directions. The first, though not clearly stated, must have originated from the impact which Darwin had on his contemporaries. Pasteur's « preconceived idea » that life of higher organisms would become impossible without bacterial symbionts is a hint in this direction. Fourteen years later (1899) Schottelius (5), an ardent admirer of Pasteur, is more articulate on the subject : « it is unthinkable that in the course of phylogenetical development some sort of symbiosis should not have developed between the host and its contaminants... » This all seems to be meant in terms of survival of the fittest, or in this adaptation, the survival of the best symbiotic systems.

The second point more clearly stated in the Duclaux-Pasteur concept refers to the germfree organism as a pure biological system. It is implied in their wording that work with such systems appears desirable. In this respect Duclaux mentions the work of Boussingault who tried to grow oats in soil watered with « pure » (distilled) water. Unfortunately, they argue, for reasons mentioned under point one, this type of experimentation is probably hopeless, because animals cannot be reared germfree. This is roughly the Duclaux-Pasteur conclusion on the matter. As mentioned before, fourteen years later Schottelius picked up this lead and fought it to the hilt (6, 7, 8). But the times changed. Shortly after the initial statement of Duclaux, the biochemists, in the infancy of their doctrine, raised their voices in protest. Analyzing intestinal contents and feces of conventional animals, they were impressed by the variety of aromatic substances which could be of bacterial origin. The veto on the theoretical field to the concepts of Pasteur came first (1886) from Nencki (9) (Switzerland). Nuttal and Thierfelder's (1895-96-97) work (10, 11, 12) on germfree guinea pigs was intended to be the experimental proof of this veto. Though the actual experiment was initiated by Nuttal and Thierfelder (10), it is clear that they themselves never developed a concept in this field. Thus the stage was set for a showdown between Schottelius and Nuttal and Thierfelder, *i.e.*, the protogonists of Pasteur and Nencki, the crux of the question being whether or not bacteria are needed in the maintenance of normal life ? In this contest around the turn of the century, Schottelius was clearly the winner, *i.e.*, they are needed. The germfree experiment was referred to by Moro (1905) (13) as the « experiment of Schottelius ».

But as frequently happens the times change again. Now (1901) it was Metchnikoff (14) who clearly lined up with the Nencki-Nuttal-Thierfelder forces. Into his world of active body defenses, the intestinal bacteria fitted in much better as fighting antagonists than as symbiotic helpers. Like Pasteur, Metchnikoff himself never performed a germfree experiment. His right hand man, Cohendy (15) answered Metchnikoff's question the best he could. By 1916 it was quite clear that chickens could live in the germfree state much beyond the age which was set by Schottelius as the « upper limit ». But Cohendy was a keen observer and went in his work much beyond Metchnikoff's original outline. The germfree animal's greater and special susceptibility to bacterial infection certainly did not escape his attention and brought up again, but was not so expressed, in a clearer form, the tool concept of the germfree animal. This was certainly a departure from the old concept which was seesawing between « is life possible without bacteria or is it not ? » Actually, Cohendy's observation of the high degree of pathogenicity of *B. subtilis* when present as a monocontaminant in chickens is as good as any modern experiment. His work and Wollman's (16) with cholera-infected ex-germfree (1922) guinea pigs shows the same type of interest.

Simultaneously, with Cohendy's coming of age, Kuster grew up in Schottelius' laboratory. His two germfree goats (17-21), together with Cohendy's chickens clearly turned the tide against Schottelius and the theory of symbiotic helpers disappears completely in its original form (1912-14). It was not revived again in any force until the theory of intestinal synthesis was needed to explain aberrances in the biochemical and nutritional picture. In Kuster's report (20) a wider scope of germfree experimentation is given : he emphasizes the importance of morphological, physiological, biochemical and immunological examination of germfree animals, thus foreshadowing the Lobund survey, in order to use them as tools. Kuster tried to initiate a survey with metabolism studies in his goats. He also recognized that germfree experimentation is a special problem of instrumentation. On this problem he has written a detailed report in the

(*) For this section I am indebted to the assistance of Dr. H. A. Gordon, Lobund Institute.

*Abderhalden Handbuch der biologischen Arbeitsmelhoden* (22). It is unfortunate that both Cohendy's and Kuster's efforts in the field of gnotobiotics was stopped by the advent of World War I. Though some of their work was published after the war (23), it was really only an abortive effort and Kuster (22) reported no postwar work.

One additional and so to speak arrested attempt in this period (1905) is that of Moro (13) from the great Viennese School of Escherich, which could have developed into a real bridge between germfree animal studies and human needs. Initiated with such zeal, his and his superior's enthusiasm must have ebbed out in view of the difficulty involved in the experimental approach. Thus history repeats itself.

In the period after World War I, other than the work of the Lobund group, was the work of Glimstedt *et al.* (24-28), Balzam (29) and Gustafsson (30, 31). All had only a transient interest in germfree life.

The first really long range, systematic program in gnotobiotics was that instituted at the University of Notre Dame in 1928 and which has continued uninterrupted to date. The motivation of this work has never been the question of whether life can exist without bacteria. Rather it is motivated by the thesis that the pure culture concept can be extended into experimental biology and medicine through the use of germfree life. Because this program has resulted in establishing the original thesis and providing adequate methodology, it may be of interest to describe the steps in the scientific program as originally conceived in 1928 and since developed (32). These are :

(*i*) The development of a satisfactory mechanical system in which the environment can be controlled and in which living organisms can be reared germfree and experimented upon without contamination. This to be accomplished on a scale large enough for steady operation and so standardized that it can be used routinely. The system to be built of a series of units which can be expanded or contracted according to need.

(*ii*) The development of a satisfactory system and methods for obtaining and rearing healthy laboratory animals into successive generations, germfree.

(*iii*) The study and description of useful, healthy experimental species in order to establish standards for comparison with experimental controls.

(*iv*) Exploration of the use of these techniques and animals in problems of importance to experimental biology and medicine, both on the theoretical and applied levels.

(*v*) Organizing a staff and a center to make the techniques and the animals available to experimental science since it seems obvious that the complications and expense of an installation built to an optimum size make such a center necessary.

Some twenty-seven years after the start of germfree work at Notre Dame, it is fair to say that an adequate mechanical system has been devised (Reyniers Germfree Systems I and II) and that the monkey (33-35), dog, cat, rabbit, guinea pigs, turkeys (36), chickens (37-39), mice (40), rats (41), can be obtained and reared germfree. The rat, mouse and chicken can be reared into successive generations germfree these being the only animals we have tried to breed to date. The germfree system has had an added and already demonstrated usefulness in chemistry, physics and biology for controlling the environment and a variety of experiments may be carried on successfully in the systems. The same germfree system has had wide usefulness in confining dangerous pathogens in experiments dealing with aerobiology (42-45) and for use in single gas systems where it is necessary to machine and assemble intricate parts in the total absence of oxygen or to perform complicated chemical experiments where such conditions are necessary.

Routines have been devised, tested and established for rearing germfree animals in satisfactory numbers which permit experiments to be planned and carried out uninterrupted by sterility failures. The chicken (37-39, 46, 47) has been characterized as has the rat but this task for other animals lies ahead. The germfree animal has been successfully used in a wide variety of studies such as the etiology of dental caries (48, 49), the synthesis of biologically labile methyl groups (50), amoeba-bacteria relationships (51), liver necrosis (52), vitamin interrelationships (46, 53), the growth effect of antibiotics (54), host contaminant relationship (55-57) and is presently being used in the study of radiation sickness, tumorigenesis, virology, hemorrhagic shock, etc.

A staff has been organized and a research institute (Lobund Institute) for the study of germfree life (gnotobiotics) was established in 1950. These facilities are presently being expanded so that scientists may take advantage of the facilities for study of their own problems. Thus, the original program has in the main been realized. The rearing of germfree animals is no longer a laboratory feat, nor the animal a curiosity, but it is an established fact and a new tool is now available. Moreover, the old question of whether life is possible without bacteria can be definitely answered. Bacteria are not necessary to animal life. That they influence and even menace this life is obvious and it becomes clearer with time that they aid the animals, but animals can live and pass into successive generations in the absence of bacteria.

## METHODOLOGY

### *Reyniers germfree system No. II* (*)

If the desideratum is to establish routines in gnotobiotics, then, it is necessary to standardize equipment. Accordingly, then, two main systems have been devised. System No. I is intended primarily for cold sterilization with germicides, either liquid or gas, and will not be considered further in this report. System No. II depends largely on sterilization with steam under pressure.

Reyniers germfree system No. II is composed of a series of units which can be fastened together in various combinations to function as a complete system in which germfree animals may be reared and used experimentally. This system is intended to be sterilized with steam under pressure as stated above. It may be used in special instances with cold sterilization but such methods are by no means as certain.

The standard units generally composing a complete system are : Rearing and holding units (RSU-400) (figures 1 and 2); REXU-200 Examining units (figures 3

(*) Manufactured and commercially available from Reyniers and Son, 3806 N. Ashland Avenue, Chicago, Ill., U.S.A.

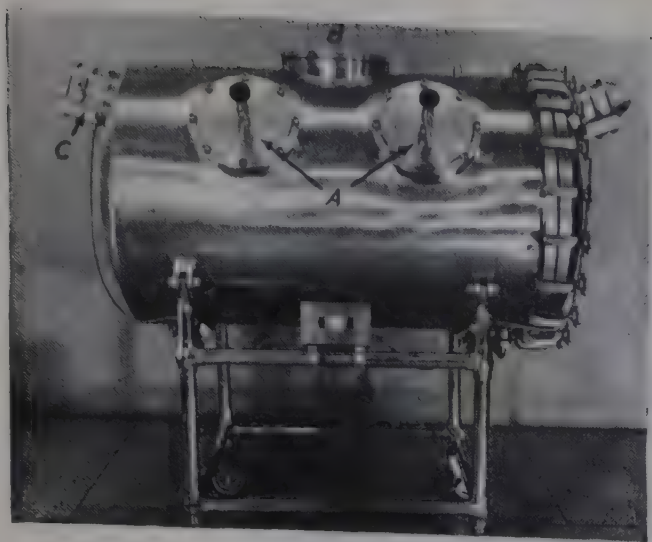

FIG. 1. — Reyniers standard germfree unit. RSU-400 (front view) *A*, glove ports with lids in place for sterilization ; *B*, viewing port ; *C*, external lamp housing.

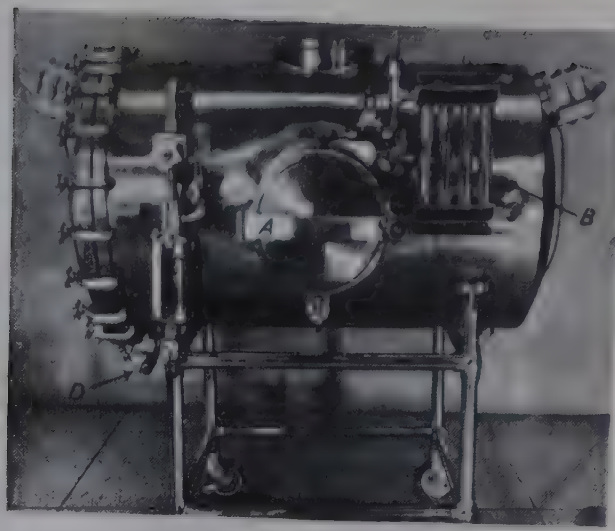

FIG. 2. — Reyniers standard germfree unit (back view). *A*, sterile lock or foor clave ; *B*, air filters ; *C*, outlet trap for air ; *D*, main drain.

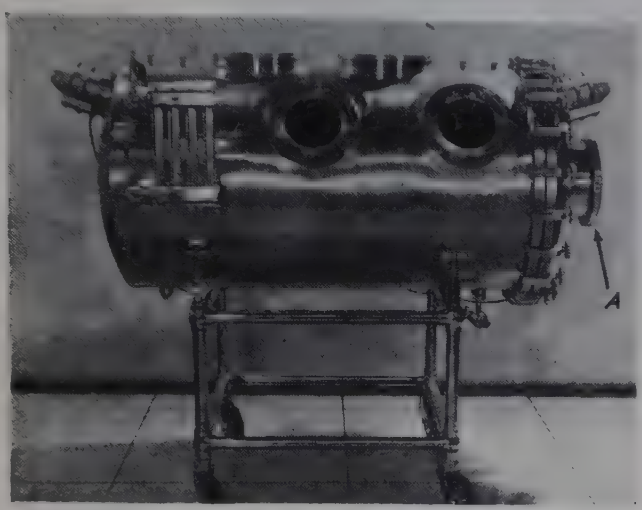

FIG. 3. — Reyniers standard examining unit. REXU-200. This is a two-man unit made to connect to an RSU-400 or other unit and is used principally for performing experiments on the animals. *A*, connecting link to sterile lock or food clave. This is equipped with a standard quick opening door to the interior.

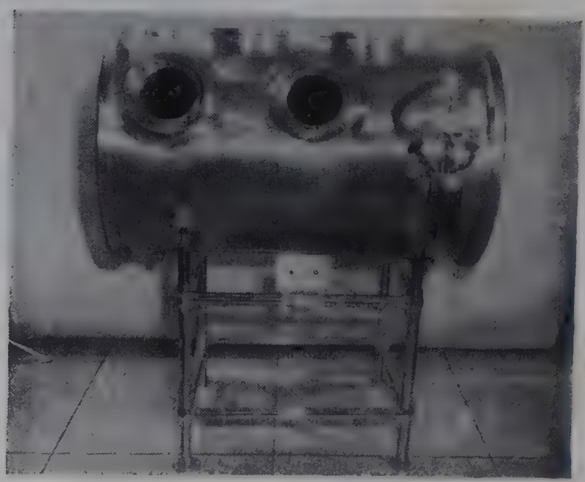

FIG. 4. — Reyniers special germfree connecting unit. REXU-200s. This is a two-man unit with two open ends and is made to butt against all other standard Reyniers units to extend the space and permit more individuals to work on the animals. This unit can also be closed off with two end doors and will then function as an REXU-200 unit.

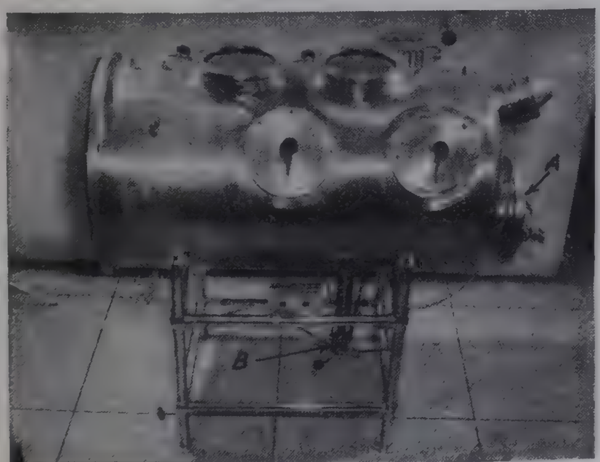

FIG. 5. — Reyniers standard surgical unit. ROPU-200. This unit is intended for surgical work, particularly Caesarian operations. This is a two-man unit made to connect to an RSU or any of the Reyniers standard units. *A*, trap for placing pregnant animal into the germfree unit. The opening to the unit is protected against contamination when the trap door is opened by a sheet of cellophane stretched across the opening. Surgery is performed by using a cautery and by burning through the cellophane. *B*, unit for raising the pregnant animal into contact with the cellophane laparotomy sheet.

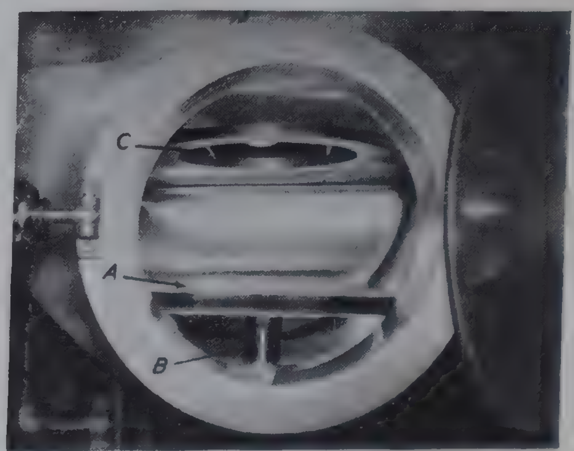

FIG. 6. — Operating trap through which pregnant animal is introduced into ROPU-200 unit. *A*, operating board ; *B*, table mechanism by which animal board is raised into contact with cellophane protective barrier ; *C*, cellophane protective barrier which separates contaminated pregnant animal from sterile interior.

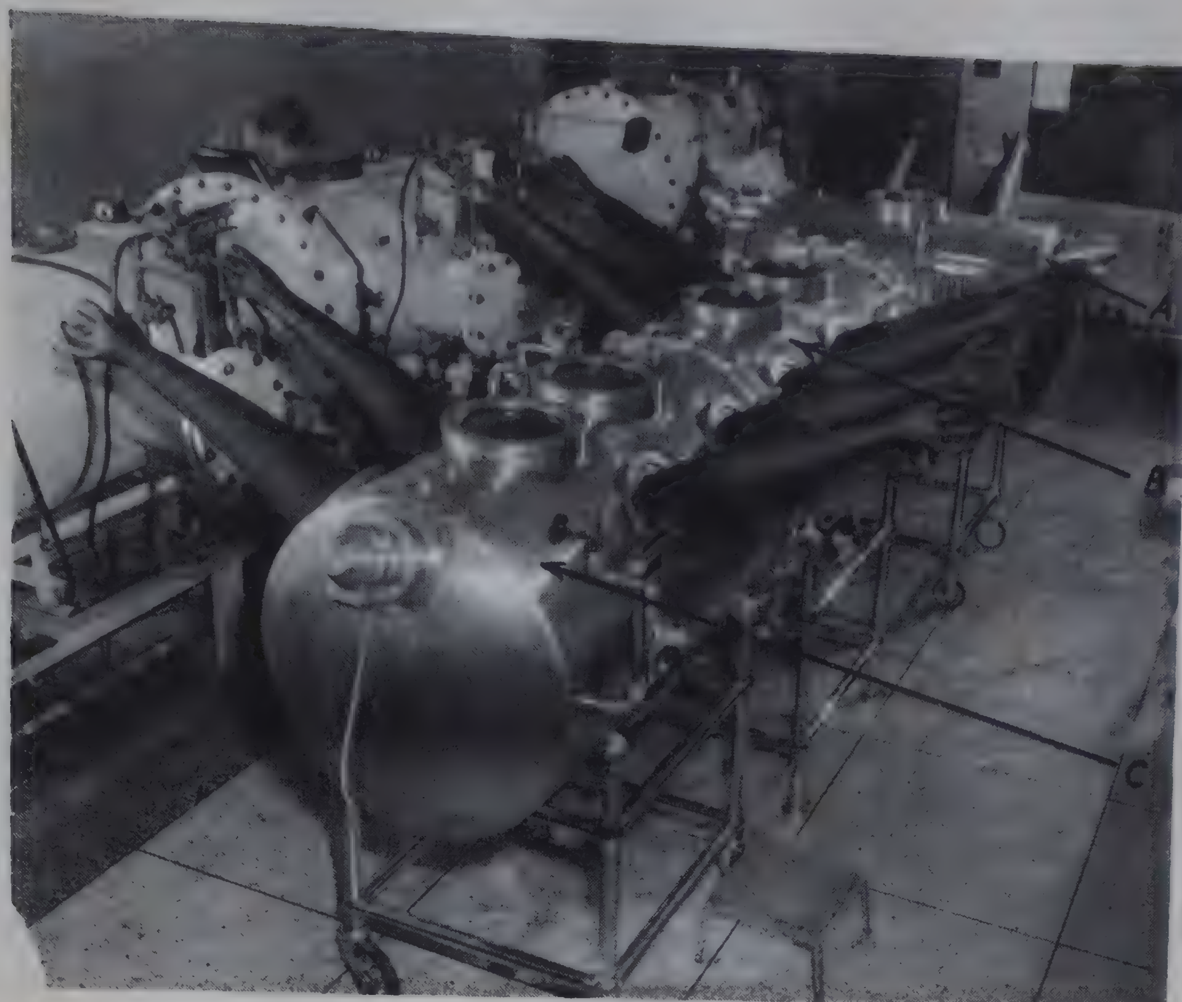


FIG. 7. — Combination of Reyniers germfree units. This combination shows three units. *A*, RSU-400 unit; *B*, REXU-200s connected to the RSU-400 *via* food clave sterile lock and to REXU-200 unit by end to end connection; *C*, REXU-200 unit.

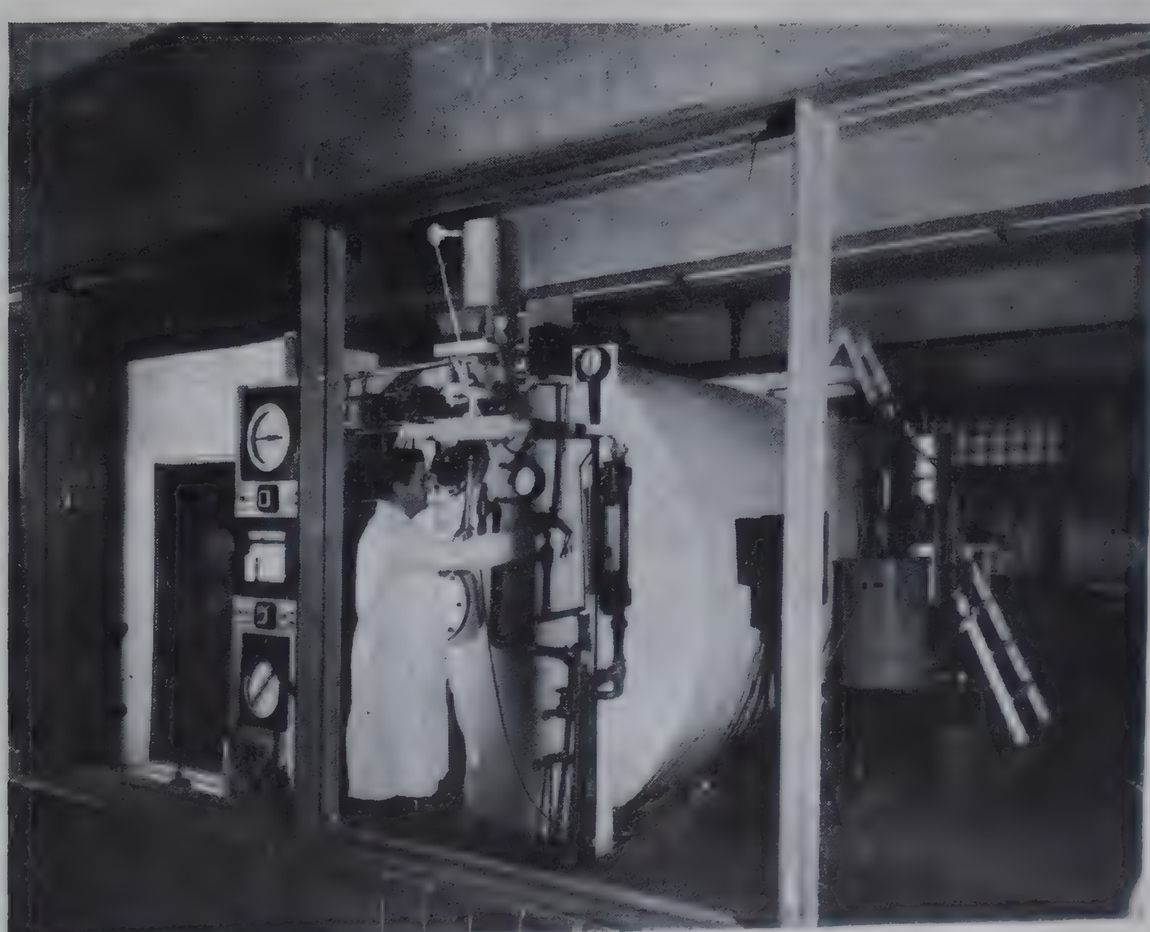

FIG. 8. — Reyniers-Trexler germfree colony unit. This unit has been in operation at Lobund Institute since 1952.

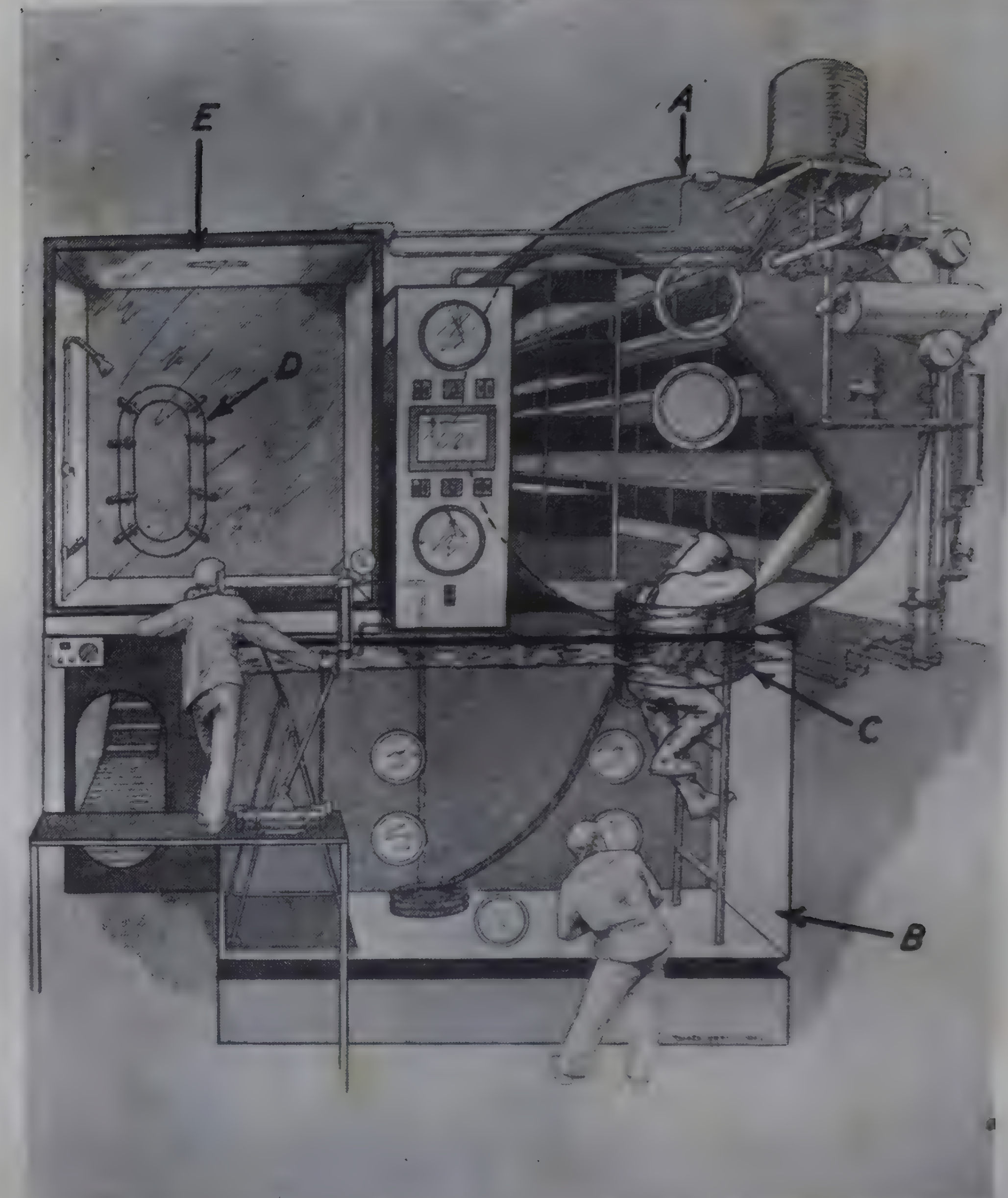


Fig. 9. — Diagram Reyniers-Trexler germfree colony unit. *A*, main colony tank ; *B*, germicidal tank ; *C*, trap to main colony tank ; *D*, entrance to dressing rooms ; *E*, germicidal shower room.

Fig. 10. — Shower room of germfree colony system. Diver clothed in plastic suit.

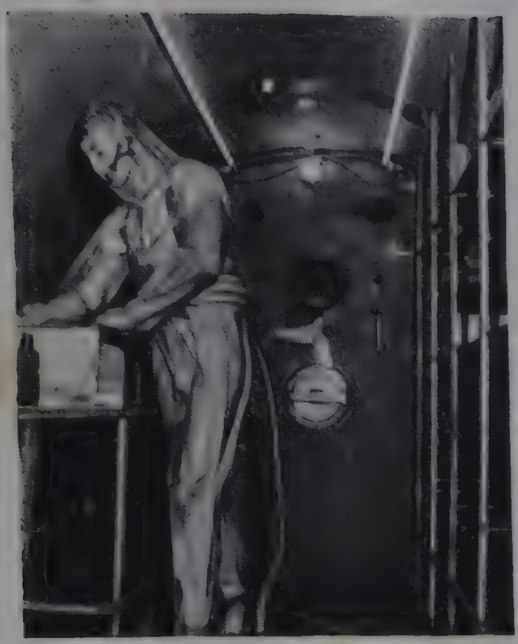

FIG. 11. — Operator in germfree colony tank.


FIG. 12. — Combination of Reyniers germfree units to form operating rearing system. *A*, operator in gloves of RSU-400 rearing unit waiting to receive Caesarian born animals *B*, operators in gloves of ROPU-200 surgical unit performing Caesarian section.

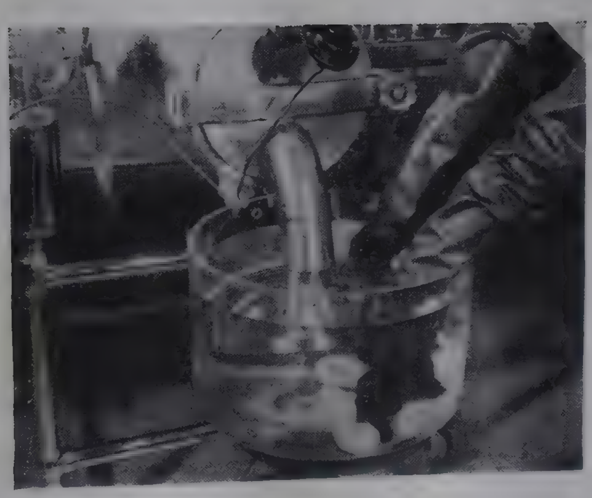

FIG. 13. — Showing germicidal trap for passing eggs into germfree system. Eggs are incubated in food clave which is equipped with thermostat and mixing valve to regulate water temperature to outer jacket of food clave.

and 4); ROPU-200 Operating or Surgical unit (figures 5 and 6).

All units are built to the same general specifications from stainless steel and vary from each other only in detail such as the number of glove openings, the overall dimensions and special functions. These units are made to attach to each other by an end to end, or sterile lock to sterile lock connection (figure 7). Only with a sterile lock to sterile lock connection can the units be separated after transfers and still maintain sterility in each unit. The end to end connection must be kept intact during the course of an experiment. The principles of this system are described (34) in the patents awarded the system.

The germfree units are made to operate from a standing position, *i.e.*, the operator must stand to use them. The height of the individual in relation to the unit is adjusted by means of platforms. The reason that this system is made to operate in a standing rather than a sitting position is obvious in that the individual has full use of his arms while standing and only from the elbows while sitting. Thus the inconvenience of standing is more than made up for by the greater reach possible. Space is always at a premium in a unit.

The diameter of all units is fixed at a standard of 28 inches (appr. 70 cm.), this being the greatest diameter which can be reached conveniently by a single operator. This diameter has in special instances been increased and instruments used to extend the reach. A diameter increase is also possible where two operators face each other. The length of the units will vary depending on the purpose and whether one or more pairs of gloves are attached. Since the gloves are always vulnerable to puncture and wear, hence contamination, it is best to avoid using more than one pair per unit, simply because the risk of contamination through a glove break is less. This is especially true where animals are to be held for long periods of time requiring many entries. The height of all units is held at 40 inches (appr. 1 m.) center line.

Viewing is through a Pyrex (Corning Glass Co.) window in top of the unit and the viewing diameter is 9 inches (appr. 22.5 cm.). The window must be ground and polished. Viewing or photography may also be accomplished through windows 4 inches (appr. 10 cm.) diameter located at either end of the germfree unit and which ordinarily serve as light sources. The number of viewing ports through which the operator looks into a unit varies with the number of pairs of gloves there being one viewing port to each pair of gloves.

Lighting the interior is accomplished by an external source in order to avoid heat load. There are two light sources for each unit located on the ends toward the top. The housing for each lamp is ventilated with a small fan and may be detached or moved aside from each glass port.

The introduction into and removal of materials from a germfree unit under sterile conditions are by way of a lock sometimes called a food clave. This is a double-walled cylinder fitted at each end with a hinged door and welded into the wall of the unit. Materials to be sterilized are placed in the food clave and subjected to steam under pressure then dried in a vacuum after which the inner door may be opened through the rubber gloves attached to a germfree unit. As mentioned before, a passage from one germfree unit to another is accomplished by a food clave to food clave connection.

The interiors of a germfree unit may be fitted with specialized animal pens or apparatus as needed. It is possible to incorporate motor driven devices, filtration apparatus, balances, metabolism cages and other laboratory apparatus inside the germfree system, the one requisite being that they can be sterilized *in situ*.

Air is supplied to the germfree system through a series of filters. The raw outside air is coarsely filtered before it is taken into the blower (oil sealed compressors cannot be used because of oil vapors) after which it may be regulated for humidity and temperature then brought to the germfree unit. At this point the air is quite free from microorganisms and dust so that there is a very light load on the final filters permanently attached to the germfree units. These final filters are filled with glass fiber packed to a specified density and are sterilized with steam under pressure and *in situ*. After sterilization the glass wool is dried with heat and in a high vacuum. In our experience this system of sterilizing air is by far the most satisfactory and simplest of any we have tried and it has been in use for fifteen years.

The gloves are the most vulnerable point in the germfree system and have been subject of much study. They are of Neoprene (Dupont), of special design and if used with care are satisfactory even after sterilization. However, no plastic or rubber membrane is puncture proof and the gloves must be handled with care at all times. They are tested for leaks by inflation.

*Reyniers-Trexler germfree colony system*

While the germfree system described above can be used for rearing germfree animals, it is limited because of space so that only a comparatively few small animals can be held in each unit. This system is more properly intended for use in experimentation, though it was clear from the beginning that a system had to be devised in which colonies of animals could be maintained through many generations. Moreover, if larger animals such as goats, dogs, etc., are to be studied, large scale apparatus is necessary. Accordingly then, after carefully considering many designs and sizes, a system was set up to accommodate a man who could work in a room or area large enough to permit standard laboratory practices for rearing and housing animals.

The germfree colony system (figures 8 and 9) finally constructed and now in operation consists of a stainless steel tank 8 feet diameter × 15 feet long (appr. 2.4 m. × 4.5 m.) connected by an entry tube dipping below the surface of a tank full of germicide 6 × 12 × 8 feet (appr. 1.8 × 3.6 × 2.4 m.) which, in turn, is connected to a germicidal spray chamber and a series of dressing rooms. The main tank and entry tube is sterilized with steam at 25 psi. The germicidal trap is filled with a mixture of 3 % formaldehyde and 1 % Roccal (Winthrop) to a level at which the entry tube is immersed.

Entry to the colony tank is effected as follows: the operator enters the first dressing room, strips and showers and dons diving underwear. He then enters a second room on the upper level and is here sealed into a plastic diving garment which is connected to a flexible tube

through which air is admitted and exhausted from the suit. This tube also carries the communication system. After a complete heat seal of the suit which is then tested for leaks, the operator enters the shower room (figure 10). Here the suit is showered with a 2 % Roccal solution followed by 1 % peracetic acid (Buffalo Electrochemical). This operation takes 20-30 minutes and with posture changes all creases and surfaces of the suit are wetted. After this operation he descends into the germicidal trap where he stays submerged, under constant observation, for 20-30 minutes. He is now ready to enter the colony tank *via* the entry tube (figure 11) where he tends the needs of the animals which are held in standard stainless mesh cages. The diver, or operator, makes no attempt to handle the animals but passes the cages through a door into a three man REX-3005-3 unit attached to the end of the colony tank. Here the bedding is changed and the animals weighed or otherwise examined.

Passage of animals into or out of the main colony tank takes place by attaching a standard RSU-400 unit to the main tank, or the REXU-300s-3 unit *via* a sterile lock passage. Food, water and other supplies are sterilized by autoclaving in the REXU-300s-3 unit where they may be sampled for sterility and hence passed into the main system.

These large scale systems bring to gnotobiotics the production of strains of germfree animals in sufficient numbers for experimental purposes. They also permit large animals to be reared and worked upon. The small scale system thus serves its intended purpose of functioning as an experimental unit. In this way a complete system for rearing breeding and using germfree animals now exists.

*Obtaining and rearing germfree animals*

Nuttal and Thierfelder (10) were the first to attempt to obtain germfree mammals by Caesarian section and birds by sterilization of the egg surface after which they could be brought into a sterile environment. This is essentially the technique in use today. The only other path, one which we have explored for many years without success, is to render a contaminated conventional animal free from contaminants.

The technique of operating and rearing germfree mammals in use at Lobund Institute has been described for mammals (34, 41) and for birds (37, 38).

Briefly, for mammals, *e.g.*, the rat timed breeding to insure the animal being at term is necessary. Also healthy disease free animals must be used for a breeding stock. Enough animals are bred to insure that some will deliver naturally between certain hours on a selected day. At this time a constant watch is set up and at the moment the rat starts to deliver she is killed by a blow on the neck delivered with a thin piece of steel. At this moment approximately ten minutes remain in which to deliver the babies alive. The dead mother is tied to a board, the surfaces painted with iodine, the animal dipped completely in a detergent and germicide. The board is now placed into a germfree operating unit ROPU-200 which is already attached to and previously sterilized with a standard rearing unit RSU-400 (figure 12). A sheet of sterile cellophane stretched over the operating field opening prevents contamination of the unit interior when the trap is opened to admit the dead pregnant animal. The dead mother is now moved up into contact with the cellophane and a midline incision is made with an electric cautery which is used throughout the operation to insure a sterile cutting instrument. The uterus is excised, the babies removed and passed into the RSU-400 unit *via* the sterile lock, the inner doors of the sterile locks closed and the rearing unit removed. It is obvious that not all animals can be killed and delivered of their young so that anesthetics are often used. In valuable animals, such as the monkey, repair is desirable and anesthetics are necessary.

Feeding the young now starts and follows a twenty-four hour schedule. Small rubber nipples are used with liquid diets. When the hand-reared animals reach maturity they may be bred and from this point on into successive generations suckle their own young — a much less tedious, and more satisfactory process.

Birds are obtained by setting clean, fertile, trap nested eggs, which are dipped into germicide before incubation begins. The germicide is permitted to dry and remains on the eggs during incubation. At twenty days embryonated eggs are obtained by candling, placed in a nylon mesh sack and passed into a sterilized rearing unit though a germicidal trap (figure 13) into the food clave where they are incubated and allowed to hatch. They are then removed to the main rearing unit.

Obtaining germfree animals is basically a matter of using clean healthy disease free stock. Such a stock colony can be obtained by using a closed animal house and originating the colony from germfree animals which are deliberately contaminated with non-pathogenic microorganisms. Such a colony has been set up and maintained for rats at Lobund Institute from time to time over the years.

## NUTRITION OF GERMFREE ANIMALS

It is well to call to mind that all animals are endowed at birth with the necessary systems and physiology to enable them to carry out their life functions and these systems exist whether an animal is born into a sterile or a contaminated world. The difference lies only in the activation of some systems or the lack of activation, depending on whether this activation is the result of an external stimulus or is a physiological ripening. Since it must be expected that the conventional animal will be endowed with systems to protect and adapt itself to microbial association and that these would not be activated *in utero* but only when the animal meets the challenge of contamination, it seems probable that these protective mechanisms will remain dormant if not called upon. Only the germfree animal can tell us if this assumption is correct.

By the same token, the diet upon which the conventional animal must subsist will be shared by its microbial flora, particularly as that flora involves the intestine simply because microorganisms are there and conditions for growth are favorable. The defensive mechanisms would be expected to play the role of confining such a flora to its location, or at best rendering the transgressors harmless when they leave the confines of the intestine. It may also be assumed that the wall of the intestinal

canal in addition to performing digestive functions will be altered by the presence of microorganisms or some of their products for purposes of microbial containment.

In the normal course of events the fermentation resulting from microbic and body activities are fairly well adapted to the economy of the animal so that at times, as in ruminants, a form of digestion may be accomplished to the benefit of the animal living on a natural diet. However, it should be pointed out that it is possible to substitute special diets which contain all the necessary elements in an available form but which are not at all dependent upon the action of microorganisms to break them down. If the study of nutrition is to be confined to the needs of the germfree animal, *i.e.*, the animal system alone, then it is the aim to provide a diet which will serve to nourish the animal even though this diet is semisynthetic and not natural to the conventional state. The role of microorganisms in the digestion and utilization of crude diets can thus be ascertained by feeding them to germfree animals. The differences in nutrition as reflected in growth between crude and artificial diets may be the sum of the contributions of the microbic flora.

In considering the nutrition of the germfree animal as such, there are, generally speaking, two major factors which must be taken into account : (*a*) all diets must be sterilized with resultant changes in the diet, and (*b*) the germfree animal has never had experience with microorganisms so that it represents a special state which must be accounted for in any extrapolation as far as this is reflected in its utilization of food.

### *Sterilization of diets*

Sterilization of diets may be accomplished by (*i*) heat, *e.g.*, autoclaving (*ii*) chemical, *e.g.*, ethylene oxide, etc., (*iii*) radiation, *e.g.*, cathode ray, (*iv*) filtration of solutions. Whatever method is used there can be no compromise with absolute sterility so that while it is possible to work at borderlines to effect sterilization, when this is done a greater reliance must be placed on testing for sterility and a greater risk of contamination accepted. Sampling of diets for sterility testing can never be as complete as feeding these diets to germfree animals because they consume all of the diet and afford a living culture medium. Unfortunately, such testing is of little value in preventing contamination.

Long experience has shown that autoclaving is the most certain of all methods of sterilization. The advantages of completely sterilizing the diet is coupled with the fact that a single *in situ* passage is possible *via* the food clave into the germfree system. It must not be forgotten that in any germfree experiment not only must the food be sterilized but also the containers and the surfaces of the sterilizing chamber through which containers are passed. Thus while it is possible to sterilize diets apart from the germfree units the problem of getting the diets into the system without contamination presents difficulties. Despite the effects of heat on diet ingredients, the effects can be overcome as has been shown by our work in which animals have been reared germfree through many generations.

Sterilization by radiation with cathode rays may also be accomplished with a reasonable degree of certainty. However, in using this method, passage into the germfree system must be accomplished *via* a germicidal trap to sterilize the containers. There are situations, however, where this risk must be taken, *e.g.*, the sterilization of heat labile materials such as some antibiotics, etc., but the method is at best adopted to experimental rather than routine operations.

The use of Carboxide (10 % ethylene oxide, 90 % $CO_2$) will produce a reasonable sterility and also permits *in situ* operation since it can be accomplished in the food clave. However, the process is slow (12-24 hours), difficult to set up mechanically because of the action of the gas on gaskets, and not without hazard of explosion. It is not readily adapted to routine operations.

If a diet or some component of the diet can be put into solution, it is possible to pass it into the germfree system through bacterial filters after which the liquid may be concentrated or reduced to solids by evaporation in a vacuum. The uncertainty of filtration as a method of sterilization, together with the mechanical difficulties involved, make this the method of choice only in special instances, *e.g.*, sterilization of sera (which does not eliminate viruses), vitamins, etc.

The nutritional adequacy of a semisynthetic diet sterilized by autoclaving or cathode ray has been shown for three generations of conventional mice (58). In our own experience steam sterilized semi-synthetic diets have permitted rats to be reared germfree through eight generations, mice through three generations and chickens through two generations which is the length of time we have elected to carry the experiments.

Ethylene oxide (59) has been shown to damage the diets used for rats and to produce toxic side effects.

* * *

In discussing the problems attendant to the rearing and nutrition of germfree animals, one can hardly ignore the rearing of Caesarian born mammals and the hand-feeding techniques necessary through the suckling period. This technique (41) with its attendant difficulties and artificialities does not enter the situation when animals are reared through successive generations germfree, naturally born and suckled by germfree mothers, but it must be faced in starting a colony of germfree animals. Moreover, the nutritional requirements of the suckling young mammals represent lacunae in the nutritional field and hand feeding techniques are both useful and necessary to fill these in. This is especially true with small mammals such as mice and rats which are wholly dependent upon the mother during the early period of life. In general, hand-feeding techniques are easier the larger the mammal and the less dependent it is upon milk, although there may be special problems with the diet. In any case, hand-feeding of a formulated milk diet is a poor substitute for natural milks and the care of the mother and is reflected in the general growth and appearance of the young. The effect of all this on the nutrition of the animal at this stage of its existence must be taken into account.

Special consideration should also be given to the fact that germfree life, as we presently understand it, has never had experience with contaminants. This is

important because it is too often presumed by those interested in this state of existence, or in using the germfree animal as an experimental tool, that it is directly comparable to the conventional animal which, of course, has had experience with contaminants since birth. The germfree animal would be more nearly comparable to the conventional animal if all contaminants could be removed from the conventional host leaving it with the experience but not the cause of this experience. However, this has never been accomplished experimentally despite constant work in this Institute for more than twenty-five years toward obtaining germfree animals in this manner. Therefore, direct comparisons between these two states of existence should be made with reservations. Furthermore, while it may seem obvious that the germfree animal and the conventional animal are endowed with the same systems and developmental experience up to the time of birth, it is only after birth and also in the case of the germfree animal passing through many generations in a sterile environment that the situation changes and the differences in environment are manifested. These facts must be kept in mind.

There are really two major paths which may be followed as far as germfree life is concerned, one is the study of the germfree animal as a unique biological state, *i.e.*, *per se* and the other is the use of germfree life as a tool in experimental biology and medicine. These paths are not necessarily mutually exclusive since it is evident that the use of germfree life as an experimental tool depends to a great extent on how well the germfree animal is characterized or known.

If the germfree state is studied *per se*, there are certain problems which manifest themselves as completely contained within this state, *e.g.*, longevity, physiology, and the study of possible symbiotic viruses and symbionts, which are unmasked in the absence of microbes and the stress they place on the animal, metabolic pathways, tumorigenesis, etc. In short, we are dealing with a pure animal, *i.e.*, an unmixed biological system throughout its life and into succeeding generations, animals which have never experienced contamination and in which the defensive mechanisms are not activated or at best are idling and held in abeyance.

If the germfree animal is used as a tool or biological model in experimental studies aimed at elucidating the conventional state ; in determining the host-contaminant relationship ; the role of microbes in the life of the animal ; in studying the various facets of infection or nutrition and the role of nutrients in metabolism and resistance to infection ; then a measure must be set up with the germfree animal at one end and conventional life at the other. The distance between is marked by the state of becoming contaminated or becoming germfree and the distance may be marked out as gradations in the process.

How far the differences between germfree and conventional animals play a role in any experiment and in particular in nutrition depends in the final analysis on the problem and the characteristics to be compared, but until these characteristics are studied and revealed by describing the germfree animal and its physiology, the validity of any comparison is questionable. Time will undoubtedly show that many organs and tissues as well as many body functions are not influenced by the presence or absence of contaminants, but we know now that some are influenced and activated by the presence of bacteria or held in abeyance by their absence. For instance, we know from our studies that animals can be obtained, reared, bred into successive generations germfree, thus fulfilling biological demands for the species studied and we know this can be done on sterilized semisynthetic diets which will also permit conventional animals to be so reared, but this does not answer the role of the intestinal flora nor does it establish the role of this flora in the life of the individual as it ages.

We know again from our studies that the intestinal canal of the germfree animal is different from that of the conventional animal. In the germfree mammal, *e.g.*, the rat for instance, the intestinal canal lacks tonus notably in the lower portion, the caecum is enlarged, the tract is much lower in lymphocytic elements, there is less framework tissue and there is less blood supply to this organ which is in a pale idling state. Dr. H. A. Gordon, my associate, has remarked (57) concerning the intestinal villi, « they are stripped to their truly functional elements ». In addition, to this, there are no serum antibodies against bacteria and the circulating phagocytes fail to take up bacteria as tested *in vitro* which simply means that these systems have not been activated (56). Yet, this intestinal canal will digest food and carry off waste as manifested by the growth of the germfree animal and the production of feces. It can also be the cause of death if the enlarged caecum develops a volvoulus. How, then, do we compare the function of the germfree intestinal tract with that of the conventional animal which has always been associated with uncontrolled contaminants, and in which great numbers of contaminants live ?

With time, as we inquire more deeply into the problem of nutrition and the biochemistry of metabolism with the attendant physiology, it is certain that these differences must be accounted for and explained. When this is accomplished, it will be by making experiments within the germfree state and as the animal is shifted toward the conventional state by adding, one by one, contaminants as pure cultures, to explain the function of the intestinal flora. It will not be accomplished by comparing the conventional animal to the germfree in one simple comparison. Therefore, in such an inquiry we might visualize the absorptive functions of the intestinal tract of conventional animals as a function which is continually burdened by the presence of active microbial elements whereas the intestinal tract of the germfree animal is not. It is by the substraction or addition of these elements that we will learn about absorption from the intestinal canal when it is left to its own resources as it is in the germfree animal. Later, by the controlled addition of these elements, we can learn about the true nature of action of the intestinal flora as it exists in the conventional animal throughout its life.

If, on the other hand, the phenomenon to be studied lies deeper in the system of the animal, *i.e.*, the internal milieu which is removed from immediate contact with contaminants, certain facts may be established in which the conditioning of the conventional animal may be compared to the unconditioning of the germfree animal.

For example, in the critical study by du Vigneaud and his associates of the synthesis of biologically labile methyl groups in the germfree rat (50), it has been shown that germfree rats are capable of synthesizing the labile methyl group and that the degree of synthesis is comparable to that observed in conventional rats. In this example the influence of contaminants within the framework of the question may make little difference.

The basic physiological functions of the intestinal canal in the germfree animal together with digestion are not, as far as we know, different from the conventional animal. By the same token the basic metabolic pathways in the tissues should be the same; all animals being endowed with these functions irrespective of the environment in which they are maintained. If alterations are found, they will probably show most clearly in time with the passage of many germfree generations and be an indication of lessened demand. For example, we have found that the first generation of monocontaminated (*Lactobacillus acidophilis*) rats demonstrated antibodies to this organism but the succeeding generations of this monocontaminated species failed to show antibodies despite the continual association of the newborn and the parents throughout their lives with this organism only. Whether this is a valid analogy for nutrition remains unanswered at this time. On the other hand, our work (47) shows that at least for the germfree chicken no new factors are needed but that deficiencies can be obtained (46) by withdrawal of individual B-vitamins. It also can recover spontaneously from vitamin K deficiency. It may be assumed, therefore, that the need for these vitamins is no different than for the conventional animal. Also, since the excreta of deficient germfree birds contain appreciable quantities of the vitamins in question which, if given orally or injected, would save their lifes, and since these vitamins are not synthesized by microbes it is probable that intestinal synthesis is relatively of less importance than originally thought. These studies were made with first generation animals, *i.e.*, not bred from germfree parents. A study of sixth generation germfree rats (53) indicated, with an occasional exception, that the germfree metabolism is very similar to its conventional counterpart. In germfree rats a source of biotin is required and less niacin, riboflavin and biotin were excreted and accumulated in body tissues than were fed. On the other hand, quantities of panthothenic acid and vitamin $B_{12}$ excreted and accumulated were equal to the amounts fed. Biosynthesis of inositol was indicated; and vitamin $B_{10}$ (measured as folic acid and citrovorum factor) was synthesized.

Admittedly these studies are incomplete as far as the questions proposed are concerned and much more systematic study is needed but the path is marked out. The need for study within the germfree state is clearly indicated. The conventional state by its very nature cannot yield an answer to the influence of bacteria in these areas without first coming through the germfree state. At best, any direct comparisons between the germfree and conventional state may be interesting but can have little direct bearing on the major problem until the base line has been established for the germfree animal.

It is essential, then, that the effect of microorganisms on the nutrition and growth of the animal be mapped out. For purposes of summary, it is evident that in the present state of our knowledge the following effects are indicated for more detailed study in the future :

(*i*) At birth and through life microorganisms may act by (*a*) altering intestinal permeability, (*b*) affecting the tonus of the tract, (*c*) changing the motility, (*d*) affecting the structure of the intestinal wall and (*e*) affecting the accessory digestive organs such as the liver.

(*ii*) Microorganisms may change the physical-chemical enrironment of the digestive tract, *i.e.*, pH, oxygen tension, fluid contents, osmotic balance, etc.

(*iii*) Microorganisms may alter the blood supply and blood elements, *e.g.*, lymphocytes to the digestive tract.

(*iv*) The nutrition of the host may be influenced by (*a*) incorporating essential nutritional elements in the microbial protoplasm, (*b*) unbalancing available and needed supplies of nutrients or metabolites, (*c*) providing nutritional elements through possible intestinal synthesis and (*d*) aiding in breaking down food stuffs such as cellulose.

(*v*) Production of toxic substances by bacteria which, if absorbed through the intestinal wall, may affect the health of the animal and, therefore, its nutrition.

(*vi*) Bacteria may condition the animal by activating body defenses and enhancing or specializing these defenses in emergencies with a reflection in the intestinal wall and possibly in the endocrine system.

(*vii*) The conventional intestinal tract may provide a reservoir of infective agents either in the intestinal canal, or in the tissues and other systems which will threaten the health of the host.

(*viii*) If the defense mechanism and reaction systems are not stressed by the action of microorganisms, there may be less burden on the animal with respect to its nutritive requirements.

Until these points are settled and standards are available for each species of germfree life, it must be evident that the use of the germfree animal in nutrition research will demand exercise of caution in interpreting the results. A great deal of study is needed yet on the germfree animal *per se* in order to establish a base line from which to work. The strains of animals must be stabilized by breeding through successive generations. The impact of contamination must be studied with all the attendant changes in structure and function. In order to accomplish these things, centers or institutes large enough to make available a steady supply of standard germfree species and the attendant equipment to keep the animals free during experimentation must be provided. In my opinion the future demands the germfree animal and the field of gnotobiotics must be developed systematically. One thing seems certain to me after almost thirty years of work in the field : there are no short cuts, no easy ways of accomplishing all this. There can be no compromise with complete control of the environment if we wish to use germfree life or to study it over extended periods of time. The technique and apparatus are expensive but there is only one sure path toward the use of the pure animal in experimental biology and that is to first study it in the absence of contaminants.

*Acknowledgement*

It is with grateful thanks that I acknowledge the contribution of my associates : P. C. Trexler, R. F. Ervin, H. A. Gordon and M. Wagner, and the help of my assistants, J. R. Pleasants and B. A. Teah.

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# Aminoaciduria in man

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(*Received* 7 *April* 1955)

The term « aminoaciduria » is nowadays used in chemical pathology to describe any situation where one or more free amino acids are encountered in the urine in larger amounts than usually occurs among normal individuals. Clearly the extent and significance of such conditions can only be assessed against a background knowledge of the quantities of these substances excreted by normal healthy subjects.

## AMINO ACIDS IN NORMAL URINE

The normal adult excretes about 1.1 g. of free amino acids daily in the urine (1). This represents about 180 mg. of nitrogen which is about 1.2 % of the total nitrogen excreted. It corresponds to about 120 mg. of α-amino nitrogen. A further 2 g. of amino acids are excreted in conjugated form (1) and these are liberated after acid hydrolysis. These are only average figures and there is much variation between individuals.

An approach to the analysis of the particular amino acids that go to make up these quantities is possible by three main methods; microbiological assay using organisms dependent for their growth on the particular amino acid to be estimated; two dimensional partition chromatography in filter paper; elution chromatography from columns of ion-exchange resins. A few individual amino acids may also be determined in urine by specific chemical methods.

Microbiological assay is not possible for a number of the amino acids present in urine because appropriate micro-organisms are not available. Furthermore the determination of free amino acids by this method may on occasion be equivocal because many amino acids are present in combined forms the microbiological availabilities of which are not known.

Two dimensional chromatography in filter paper has the advantage of simplicity and speed. The chief disadvantage is that it is at best only semi-quantitative. This method does however provide a reliable approach to the detection of most of the major abnormalities of amino acid excretion encountered in clinical medicine. It indicates qualitative disturbances in the pattern of amino acid excretion and draws attention to any unusual amino acids which may be present in quantity.

Elution chromatography from ion-exchange resins as developed by Moore and Stein (2, 3) probably represents the most satisfactory analytical method yet available. Its main drawback is the elaborate nature of the system required and the laborious estimation of the many hundreds of fractions that need to be collected.

For the isolation and subsequent characterisation of individual amino acids from urine the method of displacement chromatography from ion-exchange resins has proved of considerable value (4).

Many studies in recent years using a variety of these methods have made it clear that the amino acid composition of normal urine is very complex. Typical figures for the free amino acids in 24 hours urine specimens of normal adult males are given in table I.

TABLE I.

*Amino acids in normal urine and plasma (adult males)* (*based on* 1, 19, 11, 10, 5)

| | Urine (mg./24 h.) | | Plasma (mg./100 ml.) |
|---|---|---|---|
| | Range | Average value | Average value (post absorptive) |
| Aspartic acid | | <10 | 0.03 |
| Asparagine | 30-90 | 54 | 0.58 |
| Glutamic acid | | <10 | 0.70 |
| Glutamine | | 100 | 8.30 |
| Glycine | 70-200 | 132 | 1.54 |
| Alanine | 20-70 | 46 | 3.41 |
| Amino butyric acid | | 10 | 0.30 |
| Valine | | <10 | 2.88 |
| Leucine | 10-25 | 14 | 1.69 |
| *Iso*leucine | 10-30 | 18 | 0.89 |
| Serine | 25-75 | 43 | 1.12 |
| Threonine | 15-50 | 28 | 1.39 |
| Cysteine and cystine | 10-20 | 10 | 1.18 |
| Methionine | | <10 | 0.38 |
| Taurine | 85-300 | 156 | 0.55 |
| Proline | | <10 | 2.36 |
| Phenylalanine | 10-30 | 18 | 0.84 |
| Tyrosine | 15-50 | 35 | 1.03 |
| Tryptophan | | | 1.11 |
| Histidine | 110-320 | 216 | 1.15 |
| 1-methyl histidine | 50-210 | 180 | 0.11 |
| 3-methyl histidine | | 50 | 0.08 |
| Ornithine | | <10 | 0.72 |
| Lysine | 10-50 | 19 | 2.72 |
| Arginine | | <10 | 1.51 |
| Citrulline | | <10 | 0.50 |
| β-amino-*iso*butyric acid and β-alanine | 4-180 | 20 | 0.20 |

They are largely derived from the results of Stein (1). They were all determined by elution chromatography from the ion-exchange resin Dowex 50 with the exception of the glutamine value (5), for which this method is unsatisfactory. Even such an analysis as this is probably incomplete because amino acids excreted at levels below 5-10 mg. per day may well not have been detected. Westall (6) for example has demonstrated the presence of at least seven unknown acid-stable ninhydrin reacting amphoteric substances excreted in amounts of probably less than 10 mg. per day in normal human urine. It is interesting to note that already two new naturally occurring amino acids β-amino*iso*butyric acid (7, 8) and 3-methylhistidine (9) have been discovered by studies on normal human urine.

Side by side with the average values of the urinary amino acids in table 1 are given data obtained by Stein and Moore (10) using similar methods for the plasma levels of individual amino acids. It is evident that the relative proportions of various amino acids in the two fluids are very different. Estimates of renal clearances suggest that these are of the order of 0.5-2.5 ml. per min. for most amino acids. Glycine and histidine however have renal clearances of the order of 5-10 ml. per min. (11) while the clearance of 1-methylhistidine probably approaches the value of the glomerular filtration rate, about 120 ml. per min.

After acid hydrolysis there is considerable liberation of glycine, glutamic acid and aspartic acid from conjugates (1, 12, 13). About 70 % of the extra glycine can be accounted for as coming from hippuric acid which is excreted in quantities of 1.0-2.5 g. per day (14). The source of the rest is not yet known. The increase in glutamic and aspartic acids which are hardly present at all in the free state cannot be accounted for in terms of the glutamine and asparagine known to be present. About 50 % of the glutamic acid is however derived from phenylacetylglutamine which is normally excreted in amounts of 0.25-0.5 g. per day (14).

Many other amino acids are also liberated after acid hydrolysis though in smaller amounts (1, 12, 13). The nature of the conjugated forms from which they are derived are not in general known, though Westall (6) has recently demonstrated the presence of a number of small peptides in normal urine.

There are considerable variations in the amounts of particular amino acids excreted by different normal individuals (15, 16). The causes of this variation are not entirely understood and evidently differ from amino acid to amino acid. It is known however that differences in diet, genetical differences between individual people, and physiological changes such as pregnancy may contribute to such variations.

In general it has been found that neither the total quantity nor the distribution of the amino acids in normal urine can be correlated closely with the dietary intake of protein (2, 15, 17, 18, 19). Ten to fifteen fold increases in the dietary protein give rise in most cases to no more than a two or threefold increase in the excretion of individual amino acids. The one exception to this is 1-methylhistidine. The excretion of this substance is closely related to the amount of meat in the diet (19, 20) and it is probable that it is largely derived from the dipeptide anserine often present in quite large quantities in muscle. However, apart from 1-methylhistidine, it is clear that the variation encountered between different individuals in amount and pattern of amino acid excretion is greater than can be accounted for in terms of dietary differences (15). Even in a sample of urine collected three hours after feeding 50 g. of casein Stein, Bearn and Moore (19) found the usual amino acid pattern and quantitative values no higher than they had found in other individuals on ordinary diets.

The excretion of β-amino *iso*butyric acid appears under ordinary conditions to be little influenced by dietary variations, though it has been found that complete fasting for 2-3 days may lead to an increased excretion (21). However large differences in excretion are observed between different individuals on similar diets and these appear in the main to be genetically determined (7, 22, 23, 24). It is possible that such differences in excretion may be due to genetically determined differences in the renal tubular capacity for the reabsorption of this substance from the glomerular filtrate (7, 11).

During pregnancy there is an increase in the excretion of several urinary amino acids, histidine and threonine being the ones most markedly affected (25). Threonine increases steadily throughout gestation, whereas histidine reaches a maximum at about four months and thereafter remains at more or less the same level till term (26). During lactation the excretion levels fall rapidly often to values below those found in the non-pregnant state.

## AMINOACIDURIA IN PATHOLOGICAL STATES

During the last few years the widespread application of paper chromatography to the study of urinary amino acids has brought to light the occurrence of grossly abnormal amino acid excretion in certain conditions in which a disorder of amino acid metabolism had not previously been suspected. It is probable that further examples of such conditions remain to be discovered. Furthermore, abnormal amino acid excretion can only be assessed against the still incomplete background of what is known about the excretion of these compounds in normal individuals. It is likely that so far only gross deviations from the normal pattern of excretion have been detected and that more subtle disturbances will only become apparent when a comprehensive picture of normal excretion and its variations has been obtained.

It is useful to classify aminoacidurias into two main groups ; the « overflow » aminoacidurias, and the « renal » aminoacidurias (27, 28). The « overflow » aminoacidurias arise because there is some defect in intermediary metabolism leading to an increase in the plasma levels of one or more amino acids to such a degree that the normal renal tubular reabsorptive mechanism is unable to deal with them adequately and they therefore pass in increased quantities into the urine. The « renal » aminoacidurias are due to some defect in the processes of renal tubular reabsorption so that even at normal plasma concentrations and hence normal concentrations in the glomerular filtrate inefficient reabsorption of one or more amino acids takes place and so abnormal

amounts are found in the urine. The particular amino acids occurring in excess in the urine will depend in the « overflow » type on the specific character of the disturbance in intermediary metabolism, and in the « renal » type on the particular way in which the renal tubules are defective. It is possible, of course, that in certain circumstances both types of cause might operate, but as yet no unequivocal example of this has been demonstrated.

In general a differentiation between the two types of aminoaciduria may be obtained by a consideration of the plasma concentrations of the particular amino acids excreted in excess. More precise information is however obtained by detailed clearance studies preferably at different plasma amino acid concentrations (29). In practice this is often difficult to achieve but it becomes critical in cases where there is any doubt as to the mechanism involved, or where it is thought that both mechanism are operating.

A classification on these lines of the conditions in which aminoaciduria has been observed is given in table II. It is possible to discuss only some of these disorders in detail.

TABLE II

*'Overflow' aminoacidurias*

1. Phenylketonuria.
2. Liver disease :
   a) Massive hepatic necrosis (acute yellow atrophy).
   b) Progressive cirrhosis.
   c) Acute infectious hepatitis.

*'Renal' aminoacidurias*

3. Cystinuria.
4. Wilson's disease (hepato-lenticular degeneration).
5. Galactosaemia.
6. Fanconi syndrome.
7. « Harts » syndrome (28).
8. Organic-aciduria, decreased renal ammonia production hydrophthalmos and mental retardation (102).
9. Scurvy (103, 107).
10. Rickets (106, 107).
11. Lead poisoning (104, 105).
12. Lysol poisoning (108).

## *'Overflow' aminoacidurias*

*Phenylketonuria*. — This is a severe form of mental deficiency characterised by a gross disturbance in phenylalanine metabolism. The phenylalanine level in the plasma has been found to range between 15 and 60 mg./100 ml. (30, 31, 10). This represents on the average a thirty fold increase over the normal plasma concentration of this amino acid. There is a similar increase in the phenylalanine concentration in the cerebrospinal fluid (31). The other amino acids in the plasma and cerebrospinal fluid are present in normal amounts. Phenylalanine is excreted in the urine in quantities of 100-300 mg. per day (32, 33, 11, 10), but other amino acids are not excreted in excess. Evered (11) has estimated the renal clearance of phenylalanine in one instance and has shown that it falls within normal limits.

A number of derivatives of phenylalanine are also present in large amounts in the urine though not apparently in appreciable quantities in the plasma. These include phenylpyruvic acid, phenyllactic acid, and phenylacetylglutamine (32, 33, 14, 34).

The central abnormality in phenylketonuria appears to be a genetically determined defect in the enzyme normally concerned with the oxidation of phenylalanine to tyrosine in the liver (35, 36, 37). This is the main normal pathway in the oxidation of phenylalanine. When it is blocked, phenylalanine, derived either from the food proteins or the breakdown of tissue proteins, accumulates in large quantities in the blood and body fluids and consequently overflows into the urine.

If the patients are fed on a diet with very low phenylalanine content, the plasma phenylalanine concentration may be reduced to normal or near normal levels and the excretion of phenylalanine and its derivatives in abnormal amounts in the urine ceases (38, 39, 40, 41).

*Liver disease*. — The liver plays a key role in the deamination of amino acids and in the regulation of their plasma levels. It might therefore be expected that disorders of the liver would lead to abnormal elevation of plasma amino acid levels and hence to aminoaciduria. However, the liver possesses a considerable reserve of function in this respect and in practice gross aminoaciduria is only encountered in cases of massive liver necrosis or very advanced hepatic cirrhosis (42, 43). With less severe forms of liver damage, aminoaciduria is a much less marked finding though it undoubtedly occurs to some extent in a proportion of the patients (43, 44, 45, 46, 47). Walshe (43) who has made a detailed study by paper chromatography of urines from 119 patients with all forms of liver disease considers that the extent of the aminoaciduria runs roughly parallel with the degree of underlying liver damage both in the same patient and from patient to patient.

In comatose patients with acute hepatic necrosis there is an appreciable increase in the plasma levels of all the amino acids normally present and associated with this is a very generalised gross aminoaciduria. In most, but not all, cases of this sort the appearance of the gross aminoaciduria heralds a fatal outcome. In hepatic coma due to advanced progressive cirrhosis of the liver similar findings are obtained but in lesser degree (43).

In cirrhosis of the liver Walshe (43) emphasises the variation in pattern of amino acid excretion from patient to patient and in the same patient at different stages of the illness. Some increase in the output of most of the urinary amino acids may be detected in different cases, but cystine, β-amino*iso*butyric acid, taurine, and the amine ethanolamine are most frequently involved and appear to give the most sensitive indication of liver damage (43).

In acute infectious hepatitis about one third of the patients show a moderate aminoaciduria in the early stages of the illness, and another third give borderline values (47). When aminoaciduria is found the plasma α-amino nitrogen is raised (47).

No major abnormalities in amino acid excretion have been detected in patients with 'inactive' cirrhosis, obstructive jaundice, secondary carcinoma of the liver, or infiltrations of the liver (43).

*'Renal' aminoaciduria*

*Cystinuria*. — Patients who have a tendency to form cystine calculi in the renal tract have been found to excrete continuously in their urine large amounts not only of cystine, but also of the basic amino acids lysine, arginine and ornithine (48, 49, 50, 51). Average values obtained for the twenty four hour excretion of these four amino acids in such patients are cystine 0.73 g., lysine 1.8 g., arginine 0.83 g., and ornithine 0.37 g. (50). Other amino acids are not present in greater concentrations than occur in normal urine, though the output of taurine may be somewhat diminished (49, 50).

An abnormally high excretion of cystine may also be encountered in a number of other disorders, notably in Wilson's disease and in the Fanconi syndrome. Here however the increased cystine excretion occurs as part of a much more generalised aminoaciduria in which many aminoacids may be involved. Cystine calculus formation is hardly ever encountered in such conditions.

These various types of disorder in which an abnormal excretion of cystine is found appear to be biochemically and genetically quite distinct (49). It is convenient to reserve the term 'cystinuria' to describe the classical condition with recurrent cystine calculus formation, first described by Wollaston (53), and now known to involve an abnormal amino acid excretion peculiar to cystine and the basic amino acids.

Cystine is a relatively insoluble amino acid. In the urine between pH 5 and pH 7 it is only soluble to the extent of 300-400 mg./l. (54). The urinary concentration of a patient excreting 500-1000 mg. of cystine daily may frequently exceed this level, particularly at night time when the urine passed is most concentrated (54). Consequently the cystine will tend to come out of solution and this is presumably the prime factor in the formation of cystine calculi. The other amino acids excreted in abnormal amounts are quite soluble and hence do not lead to stone formation. In conditions such as the Fanconi syndrome and Wilson's disease although there may be an increased cystine output, the urinary cystine concentration rarely reaches levels at which it cannot be held in solution and consequently stone formation does not generally occur.

In the past cystinuria has been usually attributed to some failure in the intermediary metabolism either of cystine itself or of cysteine or methionine. This was thought to lead to an accumulation of cystine in the body and its consequent excretion in large amounts in the urine. The exact nature of the postulated block in the metabolism of the sulphur-containing amino acids was always obscure (for full reviews see 55, and 56). The discovery that lysine, arginine and ornithine were also continuously excreted in grossly abnormal amounts further complicated the issue because of the absence of any obvious specific connection between the intermediary metabolism of these amino acids and the sulphur-containing ones.

An alternative hypothesis was put forward by Dent (57), who suggested that the abnormality in cystinuria was of renal origin. The renal tubules might be unable specifically to reabsorb the cystine, lysine, arginine and ornithine present in the glomerular filtrate and these substances would therefore appear in large quantities in the urine.

The level of cystine in the blood plasma has now been investigated in a number of cystinuric patients by a variety of different methods (49, 58, 59, 60, 10). It is not elevated as would have been expected on the earlier hypothesis, but is, if anything, somewhat less than normal as might be expected on the renal theory. Similarly the plasma levels of lysine, arginine, and ornithine though not so extensively studied are apparently either within normal limits or less than normal (49, 10). Renal clearance studies in cystinurics (60) show that the cystine clearance in cystinuric patients is at least thirty times that found in normal individuals, and is of the same order of magnitude as the expected glomerular filtration rate. In one experiment where the inulin clearance and the cystine clearance were measured simultaneously, almost identical values were obtained. This indicates that little or no renal tubular reabsorption of cystine is taking place in these patients.

The relative proportions of cystine, lysine, arginine and ornithine in normal plasma have been found to be very similar to the relative proportions of these four amino acids as they occur in the urine of cystinuric patients, and this fact is also clearly consistent with the renal theory.

The results of experiments involving the feeding of cystine and cysteine to cystinuric subjects have been variously interpreted (56), but can probably be most comprehensively understood on the renal theory (59, 60). If cystine itself is fed to cystinuric patients there is little or no rise in the plasma cystine level and no increase in the urinary cystine. It appears however to be absorbed and metabolised because an appropriate increase in the urinary sulphate can be demonstrated (59, 60, 61, 62, 63). Feeding cystine to normal individuals gives substantially the same results, so that there appears to be no peculiarity in the cystinuric in respect to his ability to deal with dietary cystine.

Ingestion of cysteine by cystinurics leads to quite different phenomena. There is a rapid rise in the plasma level of cystine (or cysteine) and a very marked increase in excretion of cystine in the urine (64, 65, 66, 59, 60). If cysteine is fed to normal subjects there is a similar rise in the plasma level and also an appreciable excretion of cystine in the urine (59, 60). In general in these experiments the urinary cystine output varied with the plasma levels. However for equivalent plasma levels the cystine excretion was much greater in the cystinurics that in the normal subjects. Since the plasma tolerance curves after feeding cysteine to normal and cystinuric subjects were substantially the same, these findings. could be readily explained on the hypothesis that the renal tubular reabsorptive mechanism was defectives. This interpretation was supported by calculation of the renal clearances of cystine at different plasma levels after feeding cysteine. In cystinurics the cystine clearance did not change with increasing plasma levels but remained virtually constant at a value of the same order of magnitude as the glomerular filtration rate. In normals the clearances were very much lower but did rise with increasing plasma levels.

The reason why both in normal and in cystinuric subjects cystine ingestion fails to lead to any marked change in plasma level, whereas cysteine ingestion leads to a rapid elevation in plasma level, remains obscure. Dent and his colleagues (59) suggest that this is due to different rates of absorption probably dependent on the relative solubilities of the two substances. Cysteine being readily soluble is very rapidly absorbed through the intestinal mucous membrane, and reaches the portal blood in such high concentration as temporarily to saturate the mechanisms for maintaining a constant blood level. A large dose of cystine however will at first remain largely undissolved in the intestinal fluids and so be taken up into the portal blood only slowly and consequently be adequately dealt with by the liver. Earlier workers however (65) had postulated that there existed entirely different metabolic pathways for cystine and cysteine.

Various results have been reported after methionine feeding experiments. Earlier workers (65, 66) had found some increase in cystine excretion after feeding methionine to cystinurics, while Dent and his colleagues (59, 60) failed to find this, either in cystinurics or in normals. These discrepancies may be a result of the different time scales of the experiments.

All these experiments indicate that cystinurics do not differ in any material respect from normal subjects in their intermediary metabolism of cystine, cysteine, or methionine. However for equivalent plasma concentrations of cystine they excrete it in much greater amounts and this suggests a defect in renal function.

The renal hypothesis implies that in normal individuals the tubular reabsorption of cystine, lysine, arginine and ornithine has at least one step which is common to and specific for these amino acids, and that in cystinuria this process is in some way defective.

Cystinuria is genetically determined and examination of urine from healthy relatives of patients with cystine calculus disease has shown that many of them have an abnormal excretion of cystine and lysine and occasionally also of arginine and ornithine (52, 67, 51). Quantitative estimations of cystine, lysine, and arginine, have been performed on the urines of many such relatives of cystinuric patients and it is apparent that among them there is a very considerable variation in the excretion of these substances (51). In fact all values may be found between the very low amounts encountered in a random sample of the normal population and the very large amounts occurring in cystinuric patients with stone formation. Determinations on several samples from particular individuals over periods of up to two years showed that each person has a fairly characteristic level of excretion of these amino acids. Among these relatives of cystinuric patients there is a very high correlation between the cystine and lysine outputs (51). As the cystine rises the lysine increases *pari passu*. However abnormal arginine excretion is only consistently found when the cystine and lysine levels are relatively high. Below these levels even though the cystine and lysine outputs might well be above those found in random normal individuals, the arginine output is either within normal limits or at most only very slightly elevated (51). Apparently the cystine and lysine excretion must be above a certain threshold before arginine is excreted in excess. The same may be true for ornithine but detailed quantitative results are not yet available.

If it is true that in most cystinuric patients with stone formation there is a gross failure to reabsorb in the renal tubules cystine, lysine, arginine and ornithine, then the fact that all degrees of cystine and lysine excretion are found among their apparently healthy relatives suggests that all degrees of failure of tubular reabsorption may occur. The defect can perhaps be best visualised by considering the kind of model which Shannon (68) has suggested for glucose reabsorption by the renal tubule. One may suppose that in the normal reabsorption of cystine, lysine, arginine and ornithine, these amino acids are reversibly combined with some substance present in the renal tubule cells so that they can be taken up from the glomerular filtrate on one side of the cell and transferred to the interstitial fluid and hence the plasma on the other side. Normally enough of this substance is present to combine with virtually all the cystine, lysine, arginine and ornithine presented to it. If it is completely absent then no reabsorption will take place and all of these amino acids present in the glomerular filtrate will appear in the urine. If it is present only in limited amounts it may not be able to combine with all the cystine, lysine arginine and ornithine present in the glomerular filtrate, and so some of these substances will be excreted in abnormal amounts in the urine. If such a model is correct then it appears that arginine and perhaps ornithine have a greater affinity for the hypothetical carrier substance than have cystine or lysine, and so if the carrier substance is limited in amount, arginine and perhaps ornithine are preferentially reabsorbed.

Genetically it seems that individuals with gross cystine, lysine, arginine and ornithine excretion are homozygous for the abnormal gene involved, whereas those relatives with only moderately raised output of cystine and lysine are heterozygous, that is, possess the abnormal gene in single dose.

*Wilson's disease* (*hepato-lenticular degeneration*). — This is a rare genetically determined condition characterised by a progressive degeneration of the caudate and lenticular nuclei of the brain and by a multilobular cirrhosis of the liver. Symptoms generally appear between the ages of ten and twenty.

There is a gross disturbance of copper metabolism. At post mortem the copper content of the central nervous system and the liver is more than ten times that normally found and most of the other organs are affected in a similar way though to a lesser extent (69, 70, 71, 72). It has been shown that the patients are in positive copper balance, approximately 0.56 mg. of copper being retained per day per mg. of copper ingested (72). This appears to be due to excessive copper absorption rather than deficient elimination (72, 73). There is also a specific deficiency of the copper containing protein caeruloplasmin in the blood plasma (74), though the amount of loosely bound copper in the plasma is increased (72). The urinary copper excretion is raised

from normal values of 0-25 μg. per day to 100-800 μg. (74, 75, 76, 77).

Uzmann and Denny Brown (78) first demonstrated that a gross aminoaciduria may also occur in this condition, and this has been widely confirmed (52, 19, 79, 80). Though the majority of patients with unequivocal Wilson's disease show such an aminoaciduria, not all do so, and for this and other reasons it is thought probable that the aminoaciduria is secondary to a primary disturbance in copper metabolism (81, 72). There is also considerable variation in the degree of the aminoaciduria from case to case (19), though it is not yet clear how far this is a reflection of the progressive nature of the disease. Most of the amino acids usually found in urine may be excreted in increased amounts with the exception of taurine, 1-methylhistidine, and 3-methylhistidine (19). Proline and citrulline which are not present in appreciable quantities in normal urine are often found in quite large amounts in Wilson's disease (19). Amino acids in conjugated forms, possibly as peptides are also excreted in excess (19, 80).

The plasma amino acid content appears to be normal. The total α-amino nitrogen falls within normal limits (79, 82) and detailed quantitative studies by Stein, Bearn and Moore (19) have shown that the individual amino acids in the plasma are qualitatively and quantitatively normal, both in the fasting state and after a protein meal.

It seems probable therefore that the aminoaciduria is of renal origin and it is thought that it arises as a result of excessive deposition of copper in the kidneys and consequent damage to the renal tubules (81, 72). It is of interest that some but not all of the cases exhibit a renal glycosuria, so that the tubular lesion may affect mechanisms concerned with the reabsorption not only of amino acids but of other substances as well.

*Galactosaemia.* — In galactosaemia there is a marked inability to metabolise galactose completely. After taking galactose by mouth there is a gross elevation of the blood galactose level and a consequent excretion of galactose in the urine. Infants with this condition who are on an ordinary milk diet show a persistent elevation of the blood galactose and a more or less continuous excretion of galactose in the urine. Under these conditions they fail to thrive, their livers become enlarged, they develop cataract and they frequently die in early infancy. If they survive they are liable to show a severe degree of mental retardation. If they are fed a galactose-free diet, the blood galactose falls, galactose disappears from the urine, and their physical condition rapidly improves (83, 84, 85). It is possible that the primary lesion is an impaired ability to convert galactose-1-phosphate to glucose-1-phosphate (86).

Children with galactosaemia on a diet containing galactose excrete abnormal quantities of amino acids in their urine (87, 88, 89, 90). Studies by paper chromatography indicate that there is a relatively large increase in serine, glycine, threonine and alanine and moderate increases in glutamine, valine, leucine and tyrosine (87, 88, 89). No quantitative analyses are yet available.

If galactose is removed from the diet the aminoaciduria eventually disappears, though this may take some days (89, 90). If then galactose feeding is reintroduced, the aminoaciduria gradually develops again but some days may elapse before it is clearly apparent (89). This is in marked contrast to the galactosuria which develops immediately galactose is introduced into the diet.

When the aminoaciduria is present, the plasma α-amino nitrogen level is in most cases within normal limits (91, 90, 89), though in one instance it has been reported as elevated (92). Cusworth *et al.* (89) carried out α-amino nitrogen clearances studies in a galactosaemic child on a galactose-free diet, and then during a period when galactose was being fed. On the galactose-free diet the α-amino nitrogen clearance was normal whereas after ten days on a diet containing galactose it was distinctly raised. It thus seems likely that defective renal tubular reabsorption is the main cause of the aminoaciduria, though in view of the known liver damage it is possible that this may contribute in advanced cases. The tubular disorder could be due to a toxic effect of the raised galactose content of the blood, or to the excessive accumulation of galactose-1-phosphate within the cells.

*Fanconi syndrome (Lignac-Fanconi disease, Syndrome of de Toni, Fanconi and Debré, Cystine rickets, Cystinosis, Cystine storage disease with aminoaciduria).* — This is a complex condition usually encountered in infants and young children. It is genetically determined. There is a general failure to thrive associated with a severe form of rickets resistant to the usual anti-rachitic doses of vitamin D, chronic acidosis, polyuria, renal glycosuria, hypophosphataemia, and a marked generalised aminoaciduria. In the majority of such children there is a widespread deposition of crystals of cystine in the tissues. The many features of the condition have been extensively reviewed (93, 94). A very similar type of symptom complex but presenting as a severe osteomalacia in adult life has also been recognised (95, 52, 96, 97). In the adult cases however the deposition of cystine crystals in the tissues has not been found.

Although there is a wide variability in the clinical manifestations of this syndrome the marked generalised aminoaciduria appears to be a constant finding in both children and adults. A considerable increase in the excretion of glycine, alanine, serine, glutamine, valine, leucine, *iso*leucine, phenylalanine, lysine, cystine, arginine and proline may be encountered. Histidine, taurine, 1-methylhistidine, and β-amino*iso*butyric acid are usually not involved (98, 52, 94, 11, 92).

Bickel *et al.* (94) consider that the aminoaciduria as encountered in children with this syndrome is of the « overflow » type, because in their cases they found an increase in the plasma levels of a number of the amino acids occurring in large amounts in the urine. They regard the disease as due to some generalised disturbance in the intermediary metabolism of amino acids and this is thought to be responsible for the deposition of cystine crystals in the tissues, for the elevation of the plasma amino acid levels, and for the aminoaciduria.

Dent, however, (29, 99) has failed to find any abnormality in the plasma amino acids in a series of children with

all the features of the Fanconi syndrome including cystinosis. In particular the level of α-amino nitrogen was found to be within normal limits, and the renal clearance of α-amino nitrogen in the fasting patients was much increased. In the adult type of the syndrome the same results were obtained. Here it has been shown by elution chromatography (11) that the plasma levels of all the individual amino acids were normal and that the renal clearances of those occurring in abnormal amounts in the urine were greatly increased. It was also possible to carry out α-amino nitrogen clearance studies following casein feeding. The patients behaved in the same way as the controls as far as the elevation and subsequent fall of plasma α-amino nitrogen levels following casein feeding were concerned. However at each level of plasma α-amino nitrogen the clearances of the patients were very much greater than those of the controls (29).

Dent therefore regards the aminoaciduria in this syndrome as renal in type. It is suggested that the renal tubular defect is not confined to amino acid reabsorption but also involves other substances including glucose and phosphate. This would explain the glycosuria and the hypophosphataemia. It however provides no explanation for the cystine deposition found in the tissues of the children with this syndrome and as yet no satisfactory way of accounting for this phenomenon has been suggested.

Some support for the renal theory has come from the work of Darmady and his colleagues (100, 101) who have carried out careful microdissections of the nephrons in the kidneys from such cases. They have found a peculiar and highly characteristic abnormality of the proximal convoluted tubule. This is shorter than normal and is joined to the glomerulus by a narrow swan-like neck. This peculiar finding was made both in a child of 14 months with cystinosis and in an adult case of the Fanconi syndrome without cystinosis, so that in this respect the two kinds of case do not differ. Darmady regards these findings as probably indicating a congenital defect in structure and consequently in properties of the proximal renal tubules, but secondary renal damage cannot be absolutely excluded.

While it seems likely that the aminoaciduria in the Fanconi syndrome is renal in type, it appears possible that other tissues apart from the kidney are affected in some way and account for other features of the disease.

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## Contribution to the discussion of H. Harris' report on 'Aminoaciduria in man'

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(*Received* 5 *August* 1955)

As Dr. Harris has shown in this review, aminoacidurias can reflect a wide variety of metabolic abnormalities in man. Much further information is needed on the fundamental significance of such changes and on the biochemistry of the subject as a whole. As methods for studying aminoaciduria become more specific and more convenient, the search for genetically controlled variations in amino acid excretion becomes possible and fruitful, as Dr. Dent, Dr. Harris, and their associates have demonstrated in their investigations.

As far as work from our laboratory is concerned, a recent addition to the list of urinary constituents is tyrosine-0-sulfate. Through consideration of observations made with Dr. Crokaert and Prof. Bigwood in Brussels and by Dr. Bettelheim in Cambridge, the idea arose that at least part of the bound tyrosine in human urine found by Dr. Stein, and earlier investigators, probably was present as the 0-sulfate derivative. This has proved to be the case, and Dr. Tallan and associates (1) have found that the average daily excretion of tyrosine-0-sulfate is about 30 mg. per day, a quantity which accounts for about half of the bound tyrosine and 3 to 8 % of the ethereal sulfate of urine. In a brief survey of a few pathological urines, no striking abnormalities in the excretion of the compound have been noted.

In his introduction to this review, Dr. Harris has discussed the methods available for studying the amino acids in urine and blood plasma. He has correctly referred to the fact that our ion exchange procedures although they have advantages in terms of accuracy and resolving power, require considerable time and effort. We would be remiss, perhaps, if we did not mention the studies that we are making to render the method somewhat more convenient, even though the work on this subject is research in progress. For quite a while, we have had the feeling that it might be justified to make the procedure automatic recording. During the past year Dr. Darrel H. Spackman has built an instrument for this purpose and has had the machine in use for several months. It has proved technically feasible to record the ninhydrin color automatically. By operating the columns at higher flow rates, the time of a complete analysis can be reduced to one or two days. Although it may be a while yet before we have settled on the design which best meets the requirements for a simple and versatile instrument for this purpose, the work is far enough along to demonstrate that, if there is the need, the operation of the ion exchange columns can be rendered considerably less time-consuming by appropriate instrumentation. The column methods, however, cannot approach the speed of paper chromatography for the survey of many samples.

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## Aspects physiologiques de l'élimination urinaire des acides aminés

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(*Reçu le* 2 *août* 1955)

Dans le travail que vient de nous communiquer M. Harris, se trouve résumé l'ensemble des données les plus significatives sur les aminoaciduries cliniques : tous ceux qui suivent la littérature exceptionnellement diverse de ce domaine, auront apprécié le mérite tout particulier qui s'attache à la clarté de son exposé. Ceci ne peut surprendre dès qu'on se rappelle que M. Harris est un des tout premiers à avoir travaillé cette question, qui est sienne dans une large mesure.

Les aminoaciduries héréditaires étant les mieux définies d'entre ces affections, c'est tout naturellement de l'exploration pathologique que proviennent la plupart de nos informations sur les mécanismes normaux de l'élimination urinaire des acides aminés. Le rapport qui vient de nous être présenté me semble donc l'occasion d'examiner quelques aspects de la physiologie rénale dans leurs rapports avec l'aminoacidurie libre.

L'urine de 24 heures contient chez l'adulte normal environ 1 % de son azote total sous la forme d'acides aminés libres (1, 2). Chacun d'eux s'y trouve en quantité généralement analogue chez la plupart des sujets sains examinés (1, 3), tandis que l'élimination quotidienne de ces acides chez un sujet particulier, en bonne santé, est très peu variable (4), comme si l'excrétion urinaire des acides aminés libres était un caractère constitutionnel de l'individu. Cette vue trouve confirmation dans la connaissance due à Dent, Harris et Warren (5, 6) et plus récemment à Holzel et Komrower (7), des relations génétiques qui unissent non seulement plusieurs cas connus d'aminoaciduries pathologiques, mais encore ceux

qui, sans symptômes, n'apparaissent qu'à l'examen de l'entourage familial des premiers.

Remarquons cependant avec Soupart (8) qu'à cette constance fondamentale se superpose, chez la femme, une modulation menstruelle du débit de l'histidine. La grossesse accentue vivement l'effet du cycle fécondé, et aboutit à une histidinurie véritable qui persistera autant que la grossesse (9). Ces observations attirent bien l'attention sur le caractère dynamique de la stabilité habituelle des débits observés.

Constatons immédiatement qu'il ne se trouve pas dans l'urine d'acide aminé libre qu'on ne connaisse dans le plasma, et qu'aucune aminoacidurie n'a été rapportée où un acide aminé se soit trouvé dans l'urine à une concentration qu'on ne puisse justifier par la filtration glomérulaire. Notons aussi que les rapports des concentrations des différents acides aminés plasmatiques sont physiologiquement différents des rapports correspondants de l'urine. Le néphron doit donc réabsorber sélectivement ces substances.

L'association à certaines aminoaciduries, celle de la cystinose (10) par exemple, d'une glucosurie et d'une phosphaturie suggérait une réabsorption dans le tube contourné de premier ordre. L'observation de l'aminoacidurie du saturnisme, où sont décrites des lésions localisées du tube proximal, situe leur réabsorption dans ce dernier (11, 12, 13). Les observations de Darmady (14) sur l'atrésie proximale congénitale, en ont donné la preuve anatomique la plus significative ; il y a un an, l'école de Oliver (15, 16) apportait la démonstration histochimique directe de la réabsorption proximale des acides aminés.

On peut donc comprendre l'aminoacidurie libre du type rénal, selon la distinction déjà classique de Dent (17), comme le résultat d'un déséquilibre des fonctions glomérulaire et tubulaire proximale. Cette conception, analogue à celle qu'on connaît pour la glucosurie, introduit l'examen de ces deux fonctions.

Les images les plus récentes des capillaires glomérulaires (18, 19) montrent bien la simplicité de leur paroi, qui prolonge la basale du néphron, mécaniquement soutenue par un réseau de prolongements endothéliaux et épithéliaux.

La triple stratification décrite par Rhodin (20) est suggestive de la qualité de ses images mais n'évoque aucune structure mitochondriale : la présence rapportée d'enzymes dans les glomérules isolés (21) semble donc en rapport avec les éléments cellulaires de cet appareil, plutôt qu'avec sa paroi filtrante mince. D'autre part, le transport transcapillaire de l'urine primitive par ultrafiltration passive est établi par la concordance des mesures du volume filtré, faites au moyen de marqueurs aussi indépendants que le glucose (22), la créatinine (23) (sauf chez les Primates), l'inuline (24), le dextrane (25, 26), la polyvinylpyrrolidone (27) et le polyéthylèneglycol (28). Cet accord serait inconcevable dans l'hypothèse d'un transport glomérulaire actif. Les données physiologiques sur la corrélation de la pression artérielle à la filtration glomérulaire en fournissent une confirmation indépendante (29).

L'existence d'une membrane filtrante glomérulaire sans fonction métabolique n'implique pas nécessairement que l'urine primitive doive contenir tous les acides aminés libres du plasma. En effet, les observations de Pappenheimer (30) sur la perméabilité capillaire montrent que la vitesse de traversée des parois vasculaires par une substance en solution, dépend fortement de son poids moléculaire. Dès lors glycine et tryptophane pourront-ils parvenir dans la lumière du néphron dans le rapport de leurs concentrations plasmatiques ? Les travaux récents de Wallenius (31), et ceux, indépendants, de l'école de Pitts (26), s'accordent à admettre qu'un effet de « tamisage » n'agit sur les *clearances* glomérulaires (32) que pour des poids moléculaires supérieurs à 4000. Les acides aminés ne sont donc pas retenus ou ralentis au passage du glomérule en fonction de leur taille, mais ils pourraient l'être encore par leur charge électrostatique. En effet, les acides aminés libres du plasma sont en mélange dans un tampon à pH 7.4, en présence des protéines sanguines dont la *clearance* glomérulaire est certainement faible (32) : situation qui pourrait conduire à une filtration sélective des acides aminés (33). Or, il est impossible de déterminer directement si un tel effet électrostatique a lieu au glomérule : la ponction directe du néphron selon Richards, malgré ses perfectionnements récents et son adaptation aux recherches sur le rein de Mammifère (25, 34), ne peut fournir qu'un échantillon de quelques microlitres, très insuffisant pour le dosage significatif des acides aminés de l'urine primitive. La perfusion expérimentale du rein par du sang réfrigéré, selon la méthode introduite par Dock (35) donnerait certainement des indications intéressantes sur ce point, mais je n'ai pas connaissance qu'elle ait été utilisée à propos des aminoaciduries. Il existe cependant une raison indirecte de croire que ces effets jouent peu de rôle dans la *clearance* glomérulaire des acides aminés. Car, si on détermine quantitativement selon Moore et Stein (1, 36, 37) les acides aminés libres du plasma, en les séparant des protéines par dialyse équilibrée, on aboutit par l'analyse du dialysat à des concentrations très semblables à celles qui dérivent d'un surnageant de déprotéination picrique (38). Il est possible de rendre compte des écarts observés en fonction des erreurs expérimentales et des variations physiologiquement étroites des aminoacidémies : l'absence de déviation systématique, tant pour l'acide glutamique que pour les bases hexoniques, montre la faiblesse d'un effet électrostatique éventuel. De plus, la membrane glomérulaire ne se montrant pas colorable vitalement, on a peu de motif de la croire chargée. Si on tient compte de la *clearance* glomérulaire d'une proportion des protéines plasmatiques (32, 39), on voit que la membrane glomérulaire offre au passage des acides aminés des conditions de liberté nécessairement supérieures à celles réunies dans le dialyseur expérimental (33). En l'absence d'effet électrostatique décelé dans ce dernier, il m'apparaît que les acides aminés plasmatiques doivent franchir ensemble la paroi glomérulaire pour se retrouver dans la capsule de Bowman aux concentrations qu'ils ont dans la phase liquide du plasma.

Ce point, dont la vérification expérimentale fait défaut, me semble une prémisse nécessaire à l'étude du travail tubulaire. Cette filtration impose à tout adulte sain la réabsorption quotidienne, active ne fût-ce que par l'isotonicité du contenu du tube proximal (40, 41), de quelque 60 grammes d'acides aminés libres.

Ce travail pourrait être le fait de mécanismes transporteurs indépendamment juxtaposés et spécifiques de chaque acide aminé, puisqu'on connaît la finesse de discrimination du néphron. Ce dernier distingue les isomères optiques, les aminoacides de la série D n'étant pas réabsorbés activement (42); il transporte la 3-méthyl-histidine, tandis que la 1-méthyl-histidine partage le sort de l'inuline. Ce cas de la 1-méthyl-histidine montre que la rétrodiffusion des acides aminés doit être insignifiante.

Les expériences de Pitts (43), sur la créatinurie expérimentale du Chien, montrent cependant l'effet réciproquement compétitif de certains acides aminés avec cette substance, et entre eux. Ceci suggère l'existence de mécanismes moins nombreux, en charge de transports tubulaires communs à de petits groupes d'acides aminés. La cystine-lysinurie plaide clairement en faveur d'un tel groupage des fonctions tubulaires. Mais l'apparition de ces fonctions dans les premières semaines de la vie (2), pendant que s'achève la maturation néo-natale du rein, ne montre pas les corrélations qui confirmeraient l'existence physiologique de tels groupages. L'aminoacidurie infantile normale ne reproduit guère de configuration connue en pathologie, et son évolution aux âges croissants (44) ne montre pas les diminutions simultanées qui témoigneraient de la mise en service de tel mécanisme commun nouvellement développé.

La difficulté de mettre en évidence de tels mécanismes chez le sujet sain me semble indiquer que le groupage des fonctions de transfert, si net dans la cystinurie, appartient davantage à la physiopathologie qu'à la physiologie normale.

C'est ainsi que l'infusion intraveineuse continue de glycine, provoque chez le chien une glycinurie importante, qu'accompagne une aminoacidurie d'autant plus fournie dans le nombre et l'abondance de ses constituants que la dose injectée a été forte, et qu'elle a été rapidement administrée. Dans le plasma, on peut vérifier qu'une telle injection expérimentale, d'une heure, n'élève notablement que la glycinémie. De sorte qu'on réalise, à l'occasion d'une glycinurie par débordement, une aminoacidurie temporaire, généralisée, de type rénal, et dont le mécanisme largement compétitif est évident.

Remarquons immédiatement que l'importance des doses injectées dans ce genre d'essai (5 à 15 mg./minute/kg. de chien) conserve toute sa valeur pratique à la classification des aminoaciduries selon Dent.

Les $T_m$ d'azote α-aminé mesurés par Pitts (43), dans des expériences semblables, montrent que la généralisation fonctionnelle de cette amino acidurie exprime la redistribution des capacités de transport limitées d'un tube proximal trop sollicité.

Cet effet ne me paraît pas incompatible avec la spécificité reconnue de ces mécanismes de transport. On peut en effet concevoir que la réabsorption des acides aminés procède par des chenaux métaboliques spécifiques pour chacun d'eux, mais dont le fonctionnement dépend d'une source d'énergie commune et limitante. Les effets de groupage dépendant soit physiologiquement de coefficients analogues à ceux de Shannon (45), soit de lésions génétiques ou toxiques portant sur quelque point commun de systèmes fondamentalement spécifiques.

Une interdépendance de ce genre ne peut surprendre à propos du segment à brosse, où sont reliés de cette manière les transports du phosphate et du glucose (46) d'une part, et ceux du phosphate, de la glycine et l'alanine (47) d'autre part. Ces dernières associations évoquent vivement le syndrome de Fanconi et sa carence phosphatasique proximale, récemment confirmée par Darmady (14). Le rôle important de la phosphatase alcaline dans l'ensemble du fonctionnement proximal, suggéré en 1947 déjà par Stowers et Dent (48) semble aujourd'hui le facteur commun des activités de ce segment.

Remarquons que cette conception prévoit une relation, compétitive elle aussi, entre protéinuries et aminoaciduries.

Les données histologiques sur la protéinurie physiologique du Rat (15) montrent la coïncidence des sites du transport tubulaire proximal. Une protéinurie fonctionnelle et passagère a pu être provoquée chez le chien par une infusion massive de glycine. Une aminoacidurie de type rénal a pu être examinée chez un protéinurique de Bence-Jones aux reins cliniquement intacts.

Ces observations m'encouragent à présenter cette conception dont le caractère fragmentaire ne m'échappe cependant pas; son développement la transformera sûrement à la lumière des travaux actuels, mais je vois son rôle principal dans les expériences qu'elle suggère.

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# Biochemical effects produced by ionizing radiation

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(*Received* 28 *April* 1955)

Ionizing radiation produces an almost unlimited variety of biochemical changes, while this report has to be restricted to a few of these only.

### *Enzyme inactivation*

The observation made by Dale (1) that by irradiation with a restricted dose of roentgen rays (if not otherwise stated, irradiation in what follows will always denote exposure to roentgen rays) we can reduce the activity of the aqueous suspension of many enzymes, and that this effect can be counteracted by numerous « protective » substances (1, 2) was a very great advance.

Shortly after Dale's discovery it looked as if the study of the effect of radiation on enzymes *in vivo* would lead to an elucidation of the nature of many of the radiation lesions. It was, however, soon found that even with heavy doses many enzymes are not inactivated *in vivo* and some are even activated. In table I the effect of irradiation of some of the enzymes present in different organs is seen. Enzymes which are refractory in an organ as the liver or kidney are deeply influenced if present in the spleen, thymus or intestinal mucosa. Oxidative phosphorylation of liver mitochondria is not influenced at all even when exposing the rat to 5000 R while a dose of 50 R suffices to depress markedly this process in the mitochondria of the spleen. In these experiments a 50 R dose led after the 2 hours to an observable cell damage (3). When animals were sacrificed at 24 hours after 20 000 R, there was no appreciable decrease in the oxidation of several substrates by their liver slices. Succinate, oxalacetate, citrate, α-ketoglurate, glutamate, fumarate and malate were all oxidized at a normal rate (4). Twenty four hours after irradiation a decrease in desoxyribonuclease I (DNA-ase I) activity and an increase in desoxyribonuclease II (DNA-ase II) activity in the spleen but no change in the DNA-ase activities in the liver was observed (149). Coenzyme A level in the rat is not reduced after lethal doses.

The above results may be interpreted in different ways. One may say that the constituents of the liver tissue exercise a more effective protection than those of the spleen, or that restitution of the damage inflicted is easier executed in the liver. We may, however, seek an explanation of the above finding in the very different radiosensitivity of the liver and the spleen cell. An explanation which leads to the conclusion that it is the lesion of the cell which precedes that of the enzymes the cell harbours. It is the dislocation of the enzyme from its physiological surrounding which makes it susceptible to radiation damage.

It must be taken into consideration that it is the overall activity of the enzyme which is assayed and that such a measurement may not reveal inactivation of a minor but decisive part of the enzyme. This particular

TABLE I

*Effects of irradiation* in vivo *on the enzymes of the organs of the rat*

| Enzyme | Liver | | Spleen | | Thymus | |
|---|---|---|---|---|---|---|
| | (dose) | (effect) | (dose) | (effect) | (dose) | (effect) |
| Transaminase | 1500 R | none | 1500 R | inhibition | — | — |
| Alcaline phosphatase | 500 R | none | — | — | 600 R | increase (*) |
| Desoxyribonuclease I | 500 R | none | 500 R | decrease | — | — |
| Desoxyribonuclease II | 500 R | none | 500 R | increase | — | — |
| Succinoxidase | — | none | — | inhibition | — | — |
| Adenosintriphosphatase | — | none | 640 R | increase | 800 R | negligible |
| Oxidative phosphorylation of mitochondria | 5000 R | none | 50 R | decrease | 50 R | decrease |

(*) In bone marrow, for more then 1500 R permanent inhibition ; for 500 R increase in nuclei and nucleoli of Lieberkuhn crypts.

fraction may be located in the phase boundaries of the cellular components (5).

The Krebs cycle is also influenced in a different way by irradiation in radioresistant and in radiosensitive tissues. Fluoroacetate is known to cause an accumulation of citrate in tissues due to blocking of the citrate oxidation. In rats given a lethal X-ray dose followed by administration of fluoroacetate this combined treatment decreases citrate accumulation in spleen, thymus, ileum and pancreas but not in brain and heart, which are radioresistant organs. The effect of exposure on citrate accumulation is reversible only when a sublethal dose was administered (6). After 200 R, citric acid formation in the spleen showed an initial decrease to less than half the normal. By 14 days after 200 R, the ability of the spleen to accumulate citric acid was the same as in normal animals. After 400 R, the reversal took place at a slower rate.

In the liver of the male rat, in which no citric acid accumulation takes place after fluoroacetate administration, when irradiation takes place an accumulation is observed. The normal female rat and the male castrate can accumulate citric acid after fluoroacetate treatment. The effect of radiation here is essentially to change the metabolism with respect to the response to fluoroacetate so that the liver of the irradiated male rat resembles that of the normal female rat in its ability to accumulate citric acid. These observations suggest a hormone involvement in the radiation effect of citrate formation of the liver. Irradiation possibly interferes with the synthesis of androgenous compounds or of adrenal cortical hormones (7).

### *Haematopoietic arrest*

A beautiful example of a failure in synthesis due to disturbance of the cell in which it should have taken place is the haematopoietic arrest produced by irradiation.

Radiation anemia is the classical radiation disease. Interference with the formation of maturing erythropoietic cells in which hemoglobin is to be laid down in the course of erythrocyte formation leads to a shortage of erythrocytes in the circulation. The red corpuscles which terminated their physiological life-time are not or not fully replaced. Haematopoietic cells sustain an initial damage which is the result of interference with desoxyribonucleic acid (DNA) formation and which becomes visible when the cells enter mitosis. Six minutes only after exposure of mice to 400 R a 36 % reduction in the number of mitoses of the nucleated red corpuscles of the marrow of the mouse was observed. After 30 minutes, the decrease amounted to 77 % (8). In tadpoles exposed to 500 R haematopoietic cell destruction was found to be directly correlated with the diminished rate of cell division during the same time. When, however, massive doses of 10 000-20 000 R were applied, the rate of haematopoietic cell destruction exceeded that of cell division, indicating cell destruction independent of mitotic processes (9).

The lowest dose of roentgen rays which is stated to lead to a depressed incorporation of $^{59}$Fe into erythrocytes varies between 5 and 30 R (10, 11). Irradiation leads to a decrease in the $^{59}$Fe content of the circulating hemoglobin and to an increase in that of the blood plasma and the depot organs (12).

The lag in the effect of exposure on the incorporation of labelled iron into hemoglobin is due to the fact that hemoglobin is laid down into erythroblasts even after exposure of the animal to radiation (13). Only after the labelled erythrocytes are released into the circulation or wiped out, thus after the lapse of about a day, does the full effect of irradiation on the hemoglobin formation manifest itself.

The above observations suggest that irradiation does not interfere with hemoglobin synthesis *per se* but through interference with the *milieu* in which hemoglobin is to be synthesized.

In contrast to the incorporation of radioiron into hemoglobin and myoglobin (21), its incorporation into cytochrom *b* of the liver was not found to be depressed under the effect of exposure to up to 1300 R, nor its incorporation into catalase of the liver, when guinea-pigs were exposed to 500 R (13).

### *Cataract*

Already before any clinically visible change of the lens occurs a marked decrease of reduced glutathione and of the enzyme glutathione reductase is established. As opacity, develops, the concentration of SH-groups decreases while the activity of malic enzyme, *iso*citric and lactic dehydrogenase and cytochrome *c* reductase remains unimpaired even when opacity is fully manifest (14). In view of the specificity of the effect and the early onset of the action on the glutathione system, we are possibly faced here with a primary attack of irradiation on an enzyme system.

### *Increased uptake of labelled tissue constituents*

An increase in the formation rate of labelled tissue constituents under the effect of irradiation was repeatedly observed. Increased incorporation of acetate-2-$^{14}$C was observed into proteins of the organs of the mouse in contrast to a depressed incorporation into DNA (15). Increased incorporation of $^{14}$C into the free carboxyl groups of the aminoacids in liver protein hydrolysates was observed as well (16). After injecting acetate-2-$^{14}$C to mice, brain, fat and protein fractions were found to contain appreciably more $^{14}$C, and so did plasma and liver protein (17). An enhanced incorporation of glucose-$^{14}$C into the fats and glycogen of mouse liver was found (18). In some phases of the experiments with ascites tumor cells, increased $^{14}$C incorporation into these cells of the glycine-$^{14}$C injected mice was observed (19) and similar observations were made when studying the incorporation of $^{32}$P into phospholipids (20).

An increase in the incorporation of a radioactive tracer into a tissue constituent can be due to an increased formation rate due to additional precursors supplied through tissue destruction for example. Suppression of competing metabolic routes may have a similar effect and also a change in the sensitivity of the radioactive indicator. If, for example, we inject 1 $\mu$C of labelled glucose of negligible weight into the circulation containing 100 mg. glucose, the presence of 0.01 $\mu$C activity in a blood sample indicates the presence of 1 mg. of glucose.

Whereas, if a further amount of non-labelled glucose enters the circulation, the above statement is no longer valid as the sensitivity of the indicator is now increased.

*Interference with DNA formation*

The question which then presents itself is how the cells get damaged. In very numerous radiation lesions the highly polymerized DNA molecules are involved and we shall first discuss the effect of exposure to radiation on the formation and on the structure of these molecules.

That irradiation interferes with DNA formation was first observed when studying the incorporation of radioactive phosphate, $^{32}P$, into the DNA of rat sarcoma and of normal organs (22, 31), and later also of $^{14}C$ of acetate, glycine and other precursors into that molecule (15, 32-37). In experiments in which the incorporation of a labelled precursor into DNA extracted from organs is studied it is difficult to make sure that interference with DNA formation was not preceded by a lesion of the cell. In studies carried out on an unicellular organism, as on the ascites tumor cells, the results of which are more easily interpreted than those obtained on multicellular ones, Forssberg and Klein (36, 37) observed, however, inhibition of incorporation of $^{14}C$ of labelled glycine and also of labelled adenine into DNA extracted from these cells, the vital staining of which revealed they were living all through the 48 hours period of the experiment. When exposing the mice to 1250 R the mitotic activity of their ascites tumor cells was found to be inhibited for a period of about 15 hours whereupon mitosis slowly reappeared and reached an almost normal level after the lapse of 48 hours. In spite of the mitotic arrest and although correspondingly after the lapse of some time the number of cells in a cell sample of irradiated animals was smaller than in that of non-irradiated controls, the total mass of the two cell samples was almost identical. This was due to the fact that irradiation did not interfere with the formation of cell mass and as no new cells could be formed, the additionally formed mass accumulated in the cells already present. The size of these cells correspondingly increased. Chemical determinations revealed that, parallel with the increase in volume of the ascites tumor cells of the exposed animals, total nitrogen and pentosenucleic acid (PNA) content increased indicating that PNA and presumably most other cell constituents are synthesized at a more or less normal rate, while synthesis of DNA is almost fully inhibited. Figure 1 demonstrates the increase in the total nitrogen and PNA content of the exposed ascites cells with time in contrast to their DNA content and the same result is demonstrated by figure 2 taken from a paper of Kellner (38) who irradiated *Escherichia coli* with ultra-violet light. Following the change in the protein, PNA and DNA content of these bacteria, he found the formation of the latter to be blocked after exposure while the former continued at a remarkable rate. Irradiation by ultraviolet radiation affects DNA formation immediately. $^{32}P$ incorporation into the DNA of sarcoma of rats exposed to X-rays was investigated after the lapse of 30 minutes or more : a very pronounced effect was observed. $^{32}P$ incorporation into DNA of regenerating rat-liver (39) was, however, only found slightly depressed 3 hours after exposure to 450 R, $^{32}P$ incorporation into DNA of regenerating liver is however depressed to 50 % of that of unirradiated controls when exposing the rats to 2000 R, and similar observations were made when organs of the mouse were investigated (40). In bone marrow cultures incorporation of $^{32}P$ or

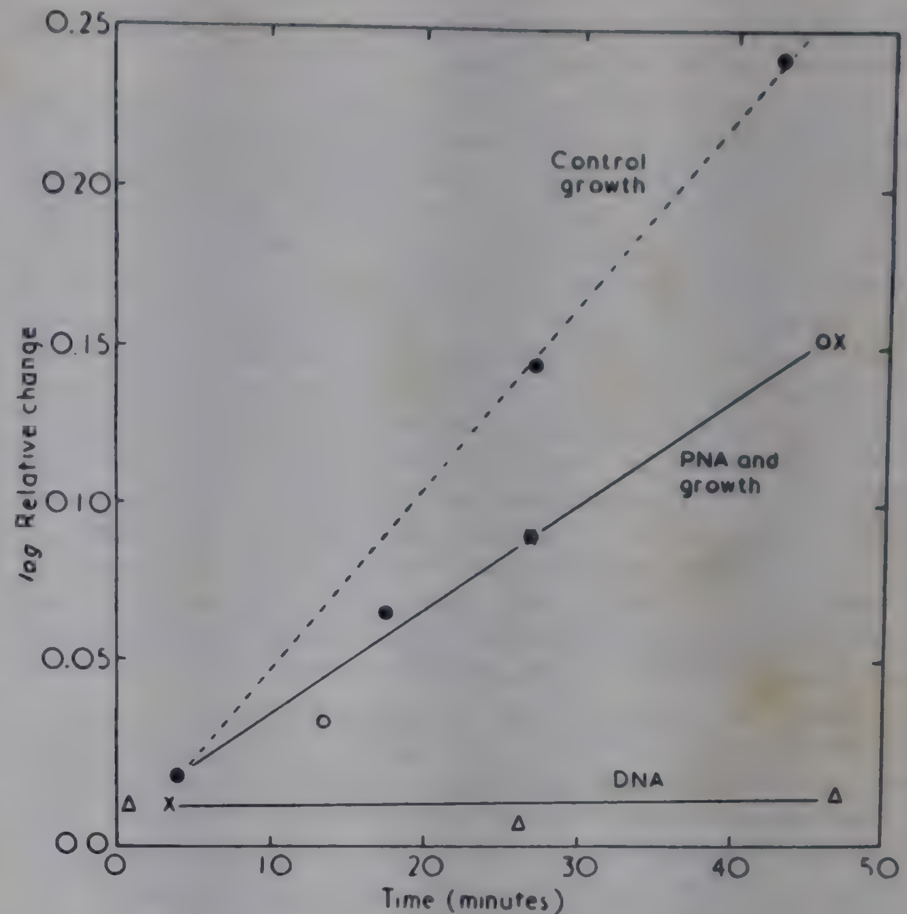


Fig. 2. — Change in the DNA and PNA content and the rate of growth of *E. coli* after irradiation with ultra-violet light.

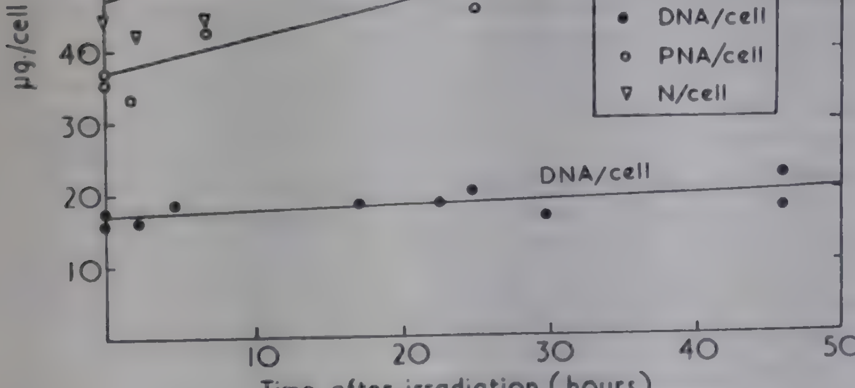


Fig. 1. — Increase of the average amount of PNA, DNA and total nitrogen per ascites tumor cell after irradiation of the mouse with 1250 R.

$^{14}C$ into DNA was immediately stopped by a 15 minutes exposure to an aggregate dose of 5000 R (41).

DNA extracted from various organs and also of the regenerating liver of rats was found to contain at least two different fractions. These differ in solubility and their ratio varies from organ to organ, they incorporate also $^{14}C$ from labelled formate at a different rate (42). While the phosphorus of two different DNA fractions of the Walker carcinoma of $^{32}P$ injected unirradiated rats had the same specific activity, they differed in the case of irradiated animals. Two hours after injection (the tumor alone was exposed to 5000 R) irradiation depressed $^{32}P$ incorporation into the 2 fractions with 52 and 60 % (43). Chargaff isolated 10 different DNA fractions and suggests the existence of possibly as much as 100 000 different fractions. These may all differ in radiation sensitivity, the measured depression of $^{32}P$ incorporation into the total DNA being a resultant of all these different interferences.

When investigating $^{32}P$ incorporation into DNA of meristem cells of *Vicia faba* making use of autoradiography Howard and Pelc have shown that incorporation takes place during an interval of about 6 hours in the course of the interphase, this process being terminated about 8 hours before the cell enters into prophase, the total time of the mitotic cycle amounting to 30 hours. A very similar result was obtained recently when investigating incorporation of $^{32}P$ or $^{14}C$ into the cells of bone marrow cultures, where the length of synthesis of DNA was found to be 12-15 hours and the total mitotic cycle 40-45 hours. In the last mentioned investigation (41) in which the radiosensitive marrow cells were exposed to 5000 R it was observed that hardly any DNA synthesis took place, if $^{32}P$ was added to the cultures 3 hours or more after irradiation, contrary to those in which the isotope was added immediately after exposure. This observation suggests that the marrow cells were damaged before entering into the DNA synthesizing phase of their mitotic cycle. In this case, inhibition of DNA formation may be a result of previous cell damage.

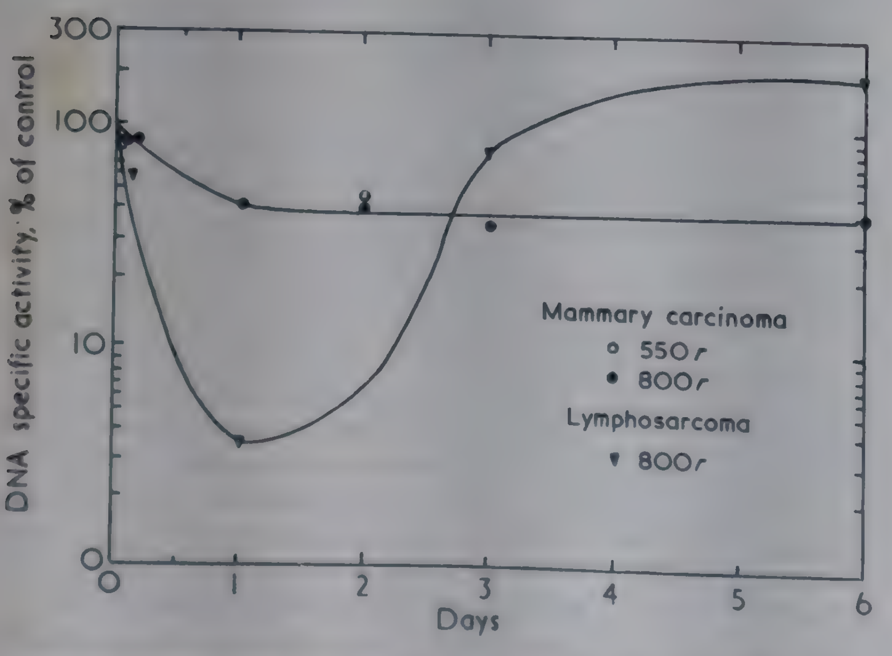


Fig. 3. — Effect of exposure on the incorporation of $^{32}P$ following intraperitoneal injection of labelled sodium phosphate (40).

While a restricted dose may produce an almost quantitative suppression of the production of additional DNA and thus of cell production even a several fold increase of the dose reduces in some cases the rate of formation of labelled, thus « new » DNA molecules, as indicated by incorporation of $^{32}P$ into the DNA molecule of the Jensen sarcoma of the rat or the mammary carcinoma of the mouse to an extent of about 50 % only as seen in figure 3.

If after the lapse of a day or so cell division will still be stopped while incorporation of $^{32}P$ into DNA continues to an extent of 50 % of that of the controls, one would have to conclude that the formation of labelled, thus « new » DNA molecules was compensated by disappearance of « old » molecules. From the fact that $^{32}P$ is incorporated into DNA in the course of the interphase only follows that the formation of labelled molecules could go on for some hours, even if the additional formation of DNA molecules is fully stopped. In the studies of Kelly (40) for example, however, 800 R depressed $^{32}P$ incorporation into DNA of mammary carcinoma by 50 %, while mitosis was fully arrested for the first 4 hours, but when investigated after the lapse of a day or later, cell division was reduced to 50 %, as was $^{32}P$ incorporation.

The problem still remains : why is even with very massive doses $^{32}P$ incorporation into DNA of the above mentioned tumors reduced to about 50 % only?

It is interesting in the above connection to note that when investigating $^{32}P$ incorporation into non-irradiated tissue it was found by several workers (23, 44, 52) that the number of additional DNA molecules formed amounts to about half of the labelled thus 'new' DNA molecules only. A possible explanation is that as about twice as many cells become labelled as formed additionally, a corresponding number of old molecules must have been removed by degradation or by exodus. The old molecules removed may have been the dividing ones which get renewed under incorporation of $^{32}P$.

This finding cannot be explained by assuming that though the number of cells does not increase their DNA content increases due to the fact that some tetraploid cells are formed, as was found in the liver of adult rodents. This may explain the minute $^{32}P$ incorporation into the DNA of the adult liver with an almost negligible mitotic figure, but not the fact that while the liver of a 3½ day old rat increases in weight and thus in DNA content by about 11 % in the course of a day, 24 % of its DNA molecules take up $^{32}P$ (24, 52).

$^{32}P$ proved to be a useful indicator in the determination of the rate of formation of DNA molecules. In fact this is the only method which permits to arrive at such data. The labelled phosphate of the circulation penetrates fairly rapidly into the cells of most tissues and gets in these in rapid exchange equilibrium with the labile phosphate of ATP which is far the most frequent phosphate donor. If, in the course of a day after administration of labelled phosphate to the animal, the mean value of the radioactivity of the labile P of ATP of the liver cells, for example, amounts to 100 and that of the P of DNA at the end of the day to 1, we can conclude that out of one hundred DNA molecules present in the liver there is one that was built up in the course of a day.

That a DNA sample is a mixture of very different types of DNA does not invalidate the conclusion arrived at

that out of 100 DNA molecules present 1 is formed in the course of the experiment, though some of these types are renewed at a different rate than others. The only objection that can be raised to this calculation is that the labile P atoms of ATP prior to their incorporation into DNA spend a comparatively long time in a phosphorus compound which is intermediary between ATP and DNA. Such an incorporation would result in a time lag of the formation of labelled DNA molecules. The radioactive indicator would not register new DNA molecules formed in the early phase of the experiment, with the result that we would underrate the speed of formation of DNA molecules.

Studying the rate of formation of DNA in human white corpuscles, Ottesen (44) found that his results can only be explained by assuming inorganic P and consequently the labile P of ATP to be the pertinent precursor of DNA. No phosphorus compound which could act as a phosphate donor is furthermore known which is formed at such a slow rate as to appreciably influence the above calculation. In spite of these facts, especially as DNA turnover may be much more rapid than in the above mentioned example, the formation rate calculated as described above is to be considered as a lower limit of that magnitude. This limitation in no way invalidates the result arrived at that the number of labelled DNA molecules is larger than the increase in the total number of DNA molecules, that with the formation of an additional DNA molecule goes the formation of about two radioactive, thus new ones. Should the calculated formation rate of labelled DNA molecules represent a lower value only, the discrepancy between the number of additionally formed and labelled DNA molecules would be still more pronounced.

The patho-chemical effects produced through interference with DNA-formation are far from being exhausted by the above statements. The exposed cell very often regains with time its faculty to synthesize DNA. The temporary inhibition of the formation of this important cell constituent, while the building-up of other constituents continues more or less undisturbed, leads often to fatal changes in the constitution of the cell which manifest themselves when the cell is to divide. As emphasized by Lea (45) and others, irradiated cells often die not at once after exposure but at a later stage after one or several divisions.

As far as cell death is the result of interference with DNA formation one would expect tissues in which DNA formation takes place at a rapid rate to be more sensitive towards exposure to radiation than tissues in which DNA is formed at a low rate only.

Interference with DNA synthesis is one of the most important, possibly the most important biochemical radiation damage. Such an interference is bound to lead to a depression of cellular multiplication in the growing organism and of the synthesis of the form elements of blood, which have to replace those parts of these elements which have reached the end of their life-cycle both in the growing and in the adult body. Furthermore, interference with desoxyribose nucleic acid synthesis may lead to anormal cells with restricted viability.

*Inhibition of mitosis not due to interference with DNA formation*

While interference with DNA formation is bound to lead to mitotic arrest, irradiation may have an inhibitive effect on other steps involved in cell division as well. Autoradiographic studies of incorporation of $^{32}P$ into DNA of cells in the root meristem of *Vicia faba* revealed that synthesis of DNA takes place in the middle of the interphase. Normal cells build up all their DNA several hours before entering mitosis and in spite of this fact are delayed by irradiation in entering division. These results indicate that irradiation can interfere with cell division not only by blocking DNA synthesis. Another possible argument for the view that irradiation can prevent cell division apart from its blocking effect on DNA synthesis are indications that the ratio of effectiveness of neutrons and roentgen rays in blocking DNA synthesis is different from that producing mitotic arrest (46).

The blocking effect of irradiation on the division of cells fully endowed with DNA may be interpreted in several ways. Energy supply necessary for transition into prophase may lack in the exposed cell for example. Mitosis cannot occur unless there is an adequate concentration of sugar and glycogen in the tissue (47). It has been shown that the depressing effect of a deficit in sugar concentration only manifests itself in the transition from resting cell stage to prophase.

The possibility must also be considered that the mechanism responsible for synthesis of DNA is involved in other steps preceding cell division as well and an interference with that mechanism or with the DNA molecules present may be responsible for the mitotic arrest produced by exposure, even in such cases in which the DNA content of the nucleus reached its predivision level.

*Radiation sensitivity*

Radiation sensitivity depends, besides from genetically determined factors, on cellular composition, on the metabolic state of the cell, the rate at which precursors and humoral agencies reach the cell and waste products are removed. In spite of the manyfoldness of factors determining radiosensitivity of an organ, a parallelity emerges between the radiosensitivity of an organ and the rate of its DNA turnover. In table II the percentage of DNA is listed which was built up under incorporation of $^{32}P$ in the course of one day together with the radiation sensitivity of the organ, ranged in decreasing order as stated by Warren and Boners (48). This statement can be completed by the observation that moderate doses were found to reduce the weight of thymus by four fifths, that of the spleen by 70 % within a few days after exposure.

A connection between turnover rate of DNA and radiosensitivity emerges in spite of the fact that each organ contains cells of different type in which DNA may be turned over at a very different rate and which correspondingly show a very different radiosensitivity. Exposure of the mouse to 400 R reduces for example the number of nucleated red cells of the marrow one day after exposure to 3 % of that of the controls, while the number

TABLE II

| DNA turnover (49-52) % per day | Organ | Radiosensitivity in decreasing order |
|---|---|---|
| | | Lymphocytes |
| 92 | Bone marrow | Erythroblasts |
| | | Myeloblasts |
| 58 | Intestinal mucosa | Epithelium of intestinal crypts |
| 42 | Thymus | Thymus |
| 7 | Spleen | Spleen |
| 2 | Muscle tissue and connective tissue | Connective tissue |
| 1 | Liver | Liver |
| 0.5 | Kidney | Kidney |
| 0.5 | Brain | Nerve and brain |
| 0 | Nucleated erythrocytes | Nucleated erythrocytes |

of total marrow cells is reduced to 58 % (53). When determining the turnover rate of the marrow DNA, the turnover rate of the DNA of the nucleated marrow cells manifests itself at a strongly reduced rate only. The connection between radiosensitivity and DNA turnover can thus be expected to manifest itself still more markedly when considering the different cell types of an organ and not the total organ. These considerations have several points in common with those put forward at a very early date by Bergonie and Tribondeau (54).

The correlation between radiation sensitivity and DNA turnover found for animal tissue stated in table II is not of universal validity. In plants for example radiosensitivity may differ by a factor of 10 or more whereas their growth rate per time unit and consequently DNA formation do not much differ. Animal lymph nodes cultivated *in vitro* showing hardly any DNA turnover were found to be fairly radiosensitive. *In vivo*, lymphocytes present in such nodes were found to be very radiosensitive (55).

One may object to the above correlation also as the radiosensitivity of tumors, for example, is far from being a simple function of their growth rates. Damage produced to the DNA synthesizing mechanism in tumors will among others be strongly influenced by blood supply. Tumors with an ample blood supply are much more radiosensitive than those lacking such supply. Furthermore, the transport of precursors, humoral substances and poisonous products may also strongly vary, influencing to a high degree both the damage produced and its restoration.

While exposure to moderate irradiation doses generally interferes with DNA formation and even often with the viability of cells, some tumor cells are not affected by such exposure. Even if an initial mitotic arrest is observed, this is soon wiped out. The possibility has been raised by Mitchell (56) that the DNA synthesis of a normal type is blocked as well in radio-resistant tumors by irradiation but that such tumor cells are endowed with the faculty to synthesise DNA following a different pathway. The possibility is also to be considered that under the numerous variety of DNA fractions such of very low radio-sensitivity are represented as well. Just like inhibition of mitosis is not necessarily due to interference with DNA synthesis, cell death may take place independantly of any disturbance of the latter. Want of oxygen or other metabolites may lead, for example, to death of unexposed cells. Circulation disturbances possibly produce these shortcomings. Such disturbances present in the normal organism may be strongly increased in the exposed one, leading to an enhanced cell death. Liver circulation, for example, was found to slow down to almost one half as a result of a local irradiation of the liver with 2300 R. E. P. (57).

### *Action on the DNA molecule*

Exposure not only interferes with DNA formation, it may lead to a rearrangement or splitting of the DNA molecule already present as well and these changes may be responsible for the blocking of the synthesis of new DNA molecules. The huge DNA molecule (m. w. = about $6 \times 10^6$) is probably assembled on a surface and if the DNA molecules present are involved in this process even a minor change in the molecular structure of the DNA molecule may vitiate the assembly of new ones. The possibility of such a template formation of DNA was repeatedly discussed (58), but even if DNA formation does not take place in a template fashion, it may still be dependent from the presence of intact DNA molecules.

*In vitro* the effect of exposure on DNA molecules can be easily demonstrated. The viscosity of DNA gels markedly decreases after irradiation with as low a dose as 25 R (59-69).

The effect of exposure on DNA *in vivo* is more difficult to demonstrate than its effect *in vitro*, as mostly high doses are to be applied to obtain an observable effect which may lead to a cell damage, and the effect observed may thus be a consequence of that damage.

Stowell (70) had to apply 4000 R to obtain a few % reduction in the DNA content of rat tumors. When the nuclei of the grasshopper were exposed to 4000-12 500 R, the Feulgen stained nuclei showed no significant loss of DNA, but the nuclei stained with methyl green disclosed a highly significant loss of stainability (71). This is interpreted to indicate that exposure to roentgen rays does not destroy the DNA but induce depolymerization of the nucleic acid.

That not only their rate of formation but the nucleoproteins *per se* are radiosensitive *in vivo* as well, is strikingly demonstrated by the following fact.

If one irradiates mature sperm in the male a genetic effect of the radiation is observed when the male is crossed with an unirradiated female. The sperm cell is not synthesizing nucleoprotein. This had been synthesized some time earlier. The fact that irradiation of the sperm nucleus does produce genetic effects demonstrates, as emphasized by Mazia, that the nucleoprotein is, in fact, radiosensitive. In a way, the chromosomal nucleoprotein appears to be the most radiosensitive of all systems.

### *Indirect action*

The primary physical act when ionizing radiation passes through water consists of the ejection of an electron from the water molecule, leaving it as a positively charged

water ion $H_2O^+$, and the attachment of the ejected electron to another water molecule, producing a negative water ion. The ensuing steps cannot be discussed in this report.

### *Oxygen action*

Most radiation actions produced by roentgen rays are enhanced by the presence of oxygen (72, 73), an observation which suggests the importance of peroxides, as these are only formed in the presence of oxygen. That the introduction of cystein and numerous other substances to some extent protects the irradiated system is interpreted as being at least partly due to their power to remove the noxious desintegration products of the water, and thus as a further proof that the radiation damage is partly due to an « indirect » effect.

A very pronounced effect of oxygen concentration on radio-damage is that found by Trowell (55). He observed that when oxygen concentration in the surrounding solution was raised from 0 to 20 ml./l., radiation damage by exposure to 3500 R of lymph nodes cultivated *in vitro* was more than tenfold increased. This is far more than observed in most other animal and plant tissues, where the presence of oxygen increases radiation damage 2 to 3 times only. An effect of oxygen on radiosensitivity of a similar magnitude as in the above mentioned case is observed by Hollaender *et al.* (74) in their study of the effect of exposure on *Escherichia coli*.

The effect of oxygen in enhancing radiation damages can also have other reasons than its role in producing peroxides. The presence of oxygen may enhance radiation effects not exclusively through enhancement of oxidative reactions, but also because the oxygen acts as a means for « storing » radicals which may later become available for reaction with sensitive systems. Investigations with *Sarcina lutea* revealed (75) that it is possible to suppress almost completely the « oxygen effect » at doses up to 26 000 R, if only cell respiration is inhibited by respiratory poisons as CO, KCN, hydroxylamin and azide.

While the very great importance of the role of the decomposition products of water in the production of radiation damages, thus an « indirect » action, can be hardly doubted, effects of exposure to radiation on tissue constituents in which these decomposition products of water are not involved, thus a « direct » action, may be of very great importance as well. A direct effect prevails when the energy is produced and released inside the molecule.

### *Direct action*

Irradiation damages produced at the temperature of liquid air are difficult to interpret as being due to an indirect action in view of the low diffusion rate of radicals at that temperature, although it is sometimes difficult to exclude the possibility that the radicals formed at low temperature are conserved and produce their damaging effect at a later stage, when, before the study of a radiation damage, the system is brought into a liquid stage.

Svedberg and Brohult (76) found haemocyanin molecules from the snail *Helix pomatia* to be split into halves to exactly the same extent, whether the solution was irradiated at 20° C. or frozen at —180° C. A molecule is split by the passage of a single α-particle. Since the probability of ionization occurring in a given part of the molecule is extremely small, we have to conclude that the splitting action can be brought about by energy absorbed anywhere within the molecule, that energy transfer occurs within this giant molecule having a molecular weight of $9 \times 10^6$. This observation and those of Alexander (77-79) on artificial high polymer macromolecules demonstrate very convincingly that the probability of a macromolecule to sustain radiation damage is greater than that of small molecules of comparable composition.

Investigating the effect of irradiation on dry polymers where radiation damage cannot be due to interaction of the decomposition products of water, Alexander has shown that the possibility to protect a system against the effect of irradiation is not a decisive test for indirect action, since he succeeded in demonstrating protection of the irradiated polymers through addition of various substances.

### *Energy transfer*

The demonstration that in macromolecules energy transfer is possible and can involve up to six c-c bonds suggests the idea that primary radiation damage may be produced in macromolecules of vital importance. The cell nucleus is known to be much more radiosensitive than the cytoplasma, the former being occupied to an appreciable extent by highmolecular desoxyribonucleoproteins. Energy absorbed by such a large molecule could be concentrated on a vital spot. Possibly under the effect of irradiation hydrogen bonds are broken, which hold the structure together. Denaturation and precipitation of proteins under the effect of exposure may be due as well to the rupture of hydrogen bonds by a direct or indirect effect. Franck and Platzman (80) have put forward the view that denaturation of protein can be achieved by splitting of hydrogen bonds, resulting from the electric field that is set up by ionization, which persists long enough and is of sufficient intensity to rupture something of the order of 30 hydrogen bonds for each ionization (81, 82). Pollard discussed recently the results of the ejection of an electron for the transfer of energy along the molecular chain.

### *Action on PNA*

While the depressive effect of irradiation on the DNA-synthesis is well established, experimental results disagree, if and in what way PNA-synthesis is influenced. Analyses of the concentration of PNA in irradiated cell samples of bacteria, ascites cells *e.g.* indicate that the new-formation of PNA proceeds rather unimpaired even when mitotic activity is inhibited.

An increase in the PNA extractable from yeast cells was observed after exposure, whereas the amount of extractable DNA was unaltered (20). This observation suggests that irradiation may cause changes in the composition and cellular distribution of PNA. That exposure of cells leads to an increase in ultraviolet absorption of the cytoplasma was observed at an early stage by Mitchell

and interpreted as due to an accumulation of pentose nucleotides in the cytoplasma (83, 84).

The results of studies on PNA-turnover applying isotopic indicators are not easy to evaluate. PNA is a heterogeneous compound as indicated by the results of Jeener and Szafarz (85) who isolated three different cytoplasmic fractions which incorporate $^{32}P$ at different rates. Nuclear PNA, as shown by Marshak (86) incorporates more $^{32}P$ than does cytoplasmic PNA. Furthermore, considerable variation occurs in the distribution of activity among the different nucleotides isolated from the liver of rats and mice but not from that of rabbits (87).

The complexity of these results is further increased by the observation (88-89) that incorporation of $^{32}P$ into cytoplasmic PNA of rat and mouse liver increases after total body irradiation in contrast to the specific activity of nuclear PNA which is found to be depressed. This dissimilarity, if proved to be a general rule, would suggest that irradiation produces a more or less general blockage of nuclear reactions. The difference between the specific activity of nuclear and cytoplasmic PNA may be due to different pathways of their synthesis. An alternative explanation is that competition for $^{32}P$ between reactions proceeding at different rates results in depressed $^{32}P$ uptake by the nuclear PNA.

Apart from the above conflicting results which may possibly be explained as due to a heterogenity of the isolated PNA, discrepancies are also evident between the results obtained in investigations in which the total labelled PNA was isolated from cells and tissues according to generally accepted procedures but where different isotopic indicators were used. While the rate of incorporation of different isotopes into DNA is found to be fairly independent of the nature of the precursor, incorporation into PNA is not (90, 91). While in the study of the effect of irradiation on DNA turnover similar results were obtained when using $^{32}P$ or $^{14}C$ as indicators, this was far from being the case when studying the effect of exposure on the turnover of PNA.

### *Action on acid soluble phosphates*

Besides the synthesis of nucleic acids numerous other cellular processes are affected by irradiation. Evidently ionizing radiation causes a disturbance of the dynamic equilibrium of the cell. Hence, a chain of reactions is initiated the pattern of which is mainly obscure. It is often found that cyclic changes occur in the biochemical functions, eventually leading to restoration of the normal state. Due regard must therefore be paid in radiobiological studies to the time elapsing after exposure.

That exposure of the organism influences ATP formation and consumption was observed in the studies of the composition of the acid soluble P fractions extracted from the skin of the rabbit (92) and from the liver of the rat (93) and in other cases as well. In the first two post-irradiation hours the ATP of ascites tumor cells of mice injected with $^{14}C$-glycine took up more $^{14}C$ than that of controls (36). A transient increase in the amount of ATP present in these experiments took place as well.

### *Action on glycogen metabolism*

While massive doses of the order of $10^5$ R cause a drastic reduction in the liver glycogen content of hamsters mice and salamanders within a few hours (94), a higher glycogen content of the liver of rats and mice fasting one day prior to irradiation to medium doses was observed one day after exposure than in the liver of fasting controls. The liver of irradiated and fasted rats and mice thus possesses the ability to accumulate liver glycogen (95-101).

Increased incorporation of $^{14}C$ of labelled glucose into liver glycogen of mice a few minutes after irradiation with 200-2 500 R was observed as well, the increase reaching a maximum after the lapse of about 1 hour, being no longer observable after a further hour. As the excess amount of $^{14}C$ deposited into glycogen of the liver of exposed mice was much higher than could be accounted for by a decreased glucose catabolism, it was suggested that a part of the $^{14}C$ incorporated into the glycogen of irradiated liver is derived from protein catabolism, which was repeatedly found to be accelerated by exposure (102).

The amount of $CO_2$ exhaled by lethally exposed mice was found to be by 10 % smaller than that exhaled by controls. When prior to irradiation labelled glucose was injected, a 20 % depression of the exhaled $^{14}CO_2$ was observed (103). With doses of 500 R temporary elevations in plasma and red corpuscle glucose were observed with a maximum increase at two to four hours from the start of the irradiation and with a return to normal within 24 hours (104).

### *Action on lipid metabolism*

A considerable amount of evidence indicates that the lipid metabolism is markedly affected by irradiation. Tracer studies indicate strong deviation from the normal incorporation rate of labelled precursors, often characterized by rapid changes in the difference of specific activity figures of the lipid fractions of exposed animals and controls (17, 18, 151). The lipid fractions of ascites cells of irradiated mice, for example, after injection of labelled glycine show very rapid changes in their activity level, which one hour after irradiation is much higher, two hours after irradiation lower than that of controls (36).

Apart from these rapid turnover changes which in most cases occur shortly after exposure, irradiation leads to an accumulation of fats in the liver and some other organs (105, 106). In fasting animals the fat reserves are decreased both in controls and irradiated animals, but much more rapidly in the former. After about 120 hours the total fat content of the control rats is down from 22 to 4 g., while the exposed animals have a fat content of 9 g. Possibly irradiation leads to a depressed fat utilization.

The plasma contains lipoproteins of differing molecular weight which are characterized by their flotation rates. The lipoproteins of the rabbit plasma are characterized by flotation rates (*) in the range $S_f$ 5 to 15. In some normal rabbits one may find in addition low concentra-

(*) One $S_f$ unit represents a flotation rate of $1 \times 10^{-13}$ cm./sec./dyne/g. in a sodium chloride solution of density 1063 g./ml. at 26° C.

tion of several of the lipoproteins in the range of $S_f$ 15 to 400. In general, higher $S_f$ rates of the lipoproteins are associated with higher molecular weights (107).

Lipids of dietary and/or endogenous sources enter into the serum lipoprotein transport pathway in the higher $S_f$ classes ($S_f$ 10 — 40 000), these are then successively transformed into lipoproteins of lower $S_f$ classes. Lipoproteins of the $S_f$ 5 to 400 group show a general increase after total body irradiation. Illustrative ultracentrifugal patterns of the serum are given in figure 4. In the 30-hour graph there is an excellent correlation between high lipoprotein levels and subsequent death of the animal. A high concentration of $S_f$ 30 to 400 lipoproteins is associated with serum opalescence. However, the $S_f$ 5 to 30 group of lipoproteins may be high enough to produce a significantly high level of total lipoproteins and the serum will remain clear. As heparin greatly accelerates the transition of high-molecular molecules into low-molecular ones, the high level of lipoproteins of $S_f$ 12 and above found in the irradiated animals is interpreted as due to a heparinemia produced by exposure.

### *Metabolic rate*

An increased metabolic rate is mostly found to increase irradiation damage. When mice are fed with dessicated thyroid, for example, for a period prior to irradiation, the death rate of these mice is markedly higher than that of animals kept on normal food (108). Seeds of plants are mostly rather radioresistant when irradiated in a dried and dormant state; when soaked in water their sensitivity increases considerably as metabolism sets in (109-111). The relative differences in the radiosensitivity

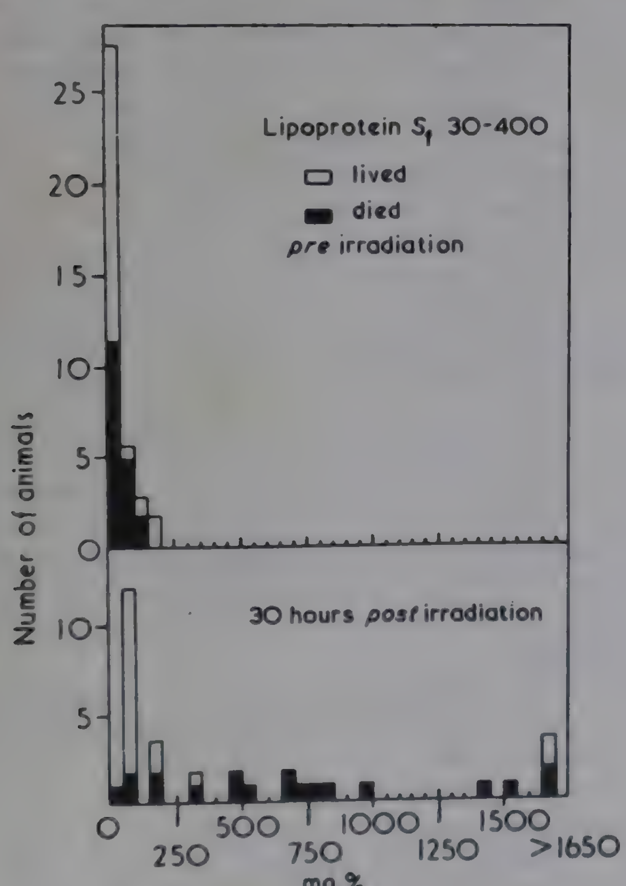


Fig. 4. — Distribution of lipoprotein concentrations before and after irradiation in 40 rabbits. Each square represents one animal.

of dry seeds of various species remain, however, almost unchanged, when these are soaked in water before irradiation.

Numerous attempts have been made to influence the radiosensitivity through variations of the temperature of the irradiated specimens shortly before, during or after exposure. The experimental evidence is difficult to evaluate and even conflicting. Whether the temperature change favours development of radiolesions more than recovery processes or *vice versa* is decisive for the result.

Maintaining of the specimens at a low temperature during irradiation in many instances is found to minimize radiation damage. Newly born mice *e.g.* irradiated at 0° C., developed in an almost normal way in contrast to those irradiated at room temperature (112). One day old rats showed much less tissue destruction when irradiated in the cold than at room temperature (113). Newborn mice have a pronounced anaerobic metabolism which is regulated through brain activity (114). It may be that cooling down of the animal correspondingly accentuates anoxia, a state in which a decrease in radiosensitivity is universally found.

In many cases decrease of temperature, however, suppresses radiation damage only temporarily, which becomes visible as soon as the temperature is raised. Marmots irradiated in a state of hibernation, for example, have about the same death rate after the completion of the hibernation period as irradiated non-hibernating marmots (115). The tissue damage produced is also approximately the same in the two groups of animals although occurring at a later stage in irradiated hibernators (116).

Ehrlich ascites cells survive freezing down to —79° C. When thawed and grafted on mice, the percentage « takes » is about the same as from grafts of ordinary, non-frozen tumor cells. Irradiation of such frozen tumor cells, in contrast to the above mentioned case, proved to be much more damaging than irradiation at room temperature as judged from graft tests (117).

### *Action on phase boundaries*

In view of the close connection which prevails between the permeability of the cell boundary and the metabolic processes going on in the cell it is very difficult to decide if for example the acceleration of the passage of $^{32}P$ into the liver cells of mice exposed to radiation (118) is due to an effect on the cell boundary or is a result of accelerated cellular metabolism. In view of the fact that the rate of incorporation of $^{32}P$ into orthophosphate and some other fractions of the liver cells of irradiated mice was found to be strongly accelerated in the exposed animal the last mentioned explanation may be more probable. It is also difficult to decide if the release of ATP by irradiated *E. coli* is due to a subtle form of lysis or a true permeability change in the cell membrane (119). Some phase boundaries remain uninfluenced even when exposed to very massive doses. After exposure to 100 000 R, the permeability of the cell membrane of *Arbacia* to water, ethylene glycol and diethylene glycol is not detectably changed (120).

A cell boundary which is certainly influenced under the effect of massive doses is the capillary wall. Eight days

after exposure to 1000 R half of the injected iodinated homologous plasma of the exposed rabbit disappears in the course of 50 min. while the loss in controls amounts to 5 % only. A similar effect of exposure was observed on the rate of loss of injected labelled red corpuscles from the circulation of the rabbit or the mouse (121). This loss following a median lethal dose is not due to a hemolysis of the erythrocytes in the circulation which are known to be very radioresistant but to an extrusion of the red corpuscles from the circulation followed by their destruction. Tagged erythrocytes were found to be lost with the same increased rate if they were introduced into the circulation before or after irradiating the rabbit. Non-irradiated erythrocytes were thus lost at an accelerated rate as well from a previously irradiated circulation.

### *Action on the reticulo-endothelial system*

Interference with the reticulo-endothelial system may contribute to the reduced formation of antibodies in the exposed organism (122-124).

The importance of bacteremia as a factor in mortality from medial radiation doses became apparent from the study of Miller and assoc. and numerous later workers in this field.

Experiments by Jacobson and Robson (125) have shown that rabbits retain the capacity to form antibody to an intravenous injection of sheep red cells if the spleen is lead-shielded during exposure up to 800 R.

### *Intestinal syndrom*

When very large doses ($LD_{100}$ at 30 days, or more) are given which produce marked intestinal syndroms the contribution of infection to mortality is much less pronounced than if smaller doses are applied. This is shown among others by the observation that even germ-free animals die after exposure to high doses (126). Within 1200 and 15 000 R the survival time of the mouse amounts to 3-5 days. This survival time was found by Rajewsky (127) not to be influenced by treatment with penicillin or streptomycin (128).

The high radiosensitivity of the abdominal region is also brought out by the observation that protection against radiation mortality is most striking when this region is shielded (129). No protection is however afforded against the component of the radiation syndroms associated with intestinal damage by administration of bone marrow or spleen homogenates which afford protection against the component of the acute radiation syndrome associated with bone marrow damage.

The severity of the intestinal syndrom after exposure to very high doses may in the first instance be due to severe changes in fluid and electrolyte balance leading to dehydration and vascular collapse and possibly also to starvation. Irradiation of rats with 1000 R causes a loss in weight of nearly identical magnitude as starvation (130). Food intake of rats is reduced by 75 % the first 24 hours after exposure to 625 R and 80-100 % the second day. Diarrhea usually appears at this time. By the fifth day it subsides and by the 8th to 10th day the bowel appears grossly normal in the surviving animal. Furthermore gastrointestinal haemorrhage is one of the most conspicuous effects of irradiation with heavy doses (131).

### *Protection*

An ever increasing amount of chemical substances has been found to afford to some degree protection against irradiation lesions when administered prior to exposure. The present status of the subject has been very comprehensively surveyed by Bacq and his colleagues (79, 132-134), who has provided much valuable research in this field. Important work, particularly on bacteria has been published by Hollaender *et al.* (135).

As a good $O_2$-supply generally favours the appearance of radiolesions, while a state of anoxia decreases the damage, one should expect that reducing substances would act as protectors. This is the case for cysteamine, cystein, gutathione, but not for ergothioneine or pantetheine (79) or for N.S.-diacetylcystein, N.S.-diacetylcysteamine and several other substances (136). An effective protector is coal gas thanks to its CO-content which produces anoxia when 60 % or more of the haemoglobin content of mice are converted into carboxy-compound (137, 138).

Lack of space prevents us from discussing this important branch of radiobiology more in detail.

### *Recovery from radiation damage*

Administration of some humoral substances are instrumental in accelerating or producing recovery.

Presumably some connection exists between the fact that spleen and bone marrow are particularly affected by irradiation and the finding that shielding of the spleen during irradiation or alternatively an injection of cell suspensions of spleen or bone marrow enhance the survival rate of exposed mice, even when administered following irradiation. Already about 1-3 $\times$ $10^6$ cells are sufficient to afford an appreciable increase in the survival rate of mice given 750-900 R (139).

The active principle was found to be bound to nuclei, all other fractions (mitochondrial, microsomal and supernatant) being ineffective. The active factor appears to be non-dialyzable, unstable, heat-labile, radiosensitive. It is susceptible to DNA-ase and trypsin but resistant to PNA-ase. The factor appears to be specific in origin ; it is found in mouse spleen, and possibly in bone marrow, but apparently not in thymus nor in liver, muscle or lung (140). Bone marrow obtained from donor mice irradiated with 200 R one day before removal of the bone marrow cells was found to have conserved its protecting power, not so bone marrow removed from mice exposed to 400 R (139).

Recently, the administration of alkoxylglycerols, particularly of batylalcohol which is present in the lipids of bone marrow and spleen was shown to decrease significantly the mortality of mice (141). This compound proved also useful in the therapy of human irradiation leucopenia. The increase in white cell counts was in most cases prompt and striking (142).

Hollaender *et al.* (143) have isolated a substance from the spleen which supports the restoration of irradiated bacteria. The factor, which is possibly identical with a

similar compound isolated from yeast appears to be soluble in acid and alcohol and to be heat-resistant, it has a molecular weight of 20 000-40 000. The amount obtained from 200 kg. of spleen was 1 g. Its chemical structure is unknown but it appears not to be related to nucleic acids.

Periston, a polyvinylpyrolidon, which has been recommended as an antidote in toxic conditions has recently also been claimed to neutralize X-ray damage after irradiation. In rats given a $LD_{50}$ at 30 days, three injections of Periston on the days following upon irradiation were found to decrease mortality to about 7 % of that of the controls. The urine from irradiated and Periston treated rats was found to be poisonous to normal rats indicating a release of some polyvinylbound deleterious compound (144). These results were not corroborated by other investigators and need further elucidation.

### *Radiosensitizers*

As already mentioned it is a general experience that the radiosensitivity of cells depends on the oxygen concentration prevailing within these. Complete anoxia reduces the radiation effect (killing of cells or the organisms, production of mutations, cytological damages as chromosome breaks and several other effects) by a factor of about 2 or 3.

Surveys of this field and many new contributions as well are given by Hollaender (135) working with microorganism as *E. coli* and by Gray (145). The latter has also tackled the important problem, whether the radiosensitivity of tumors relative to that of the normal tissue can be increased through $O_2$-inhalation to the advantage of the radiotherapist. For the Ehrlich tumor of mice it was demonstrated that inhalation of oxygen enhances the X-ray damage without causing any significant increase of the normal tissue lesions.

Promising attempts to improve the conventional methods of radiotherapy were made by Mitchell for a great number of years (146). He succeeded in showing that substances like 2-methyl-1, 4-naphthohydrochinone diphosphate (Synkavit) is selectively concentrated in tumors and that intravenous injection of this compound into patients with bronchial carcinoma markedly enhances the results of roentgen therapy. Acetylpantethein and alanine were found to increase the radiosensitivity of the mouse (150).

Recovery can also be achieved in several other ways. Irradiated *Ascaris*, for example, which is a facultative anaerobe, has a longer life-time when kept in absence of oxygen after exposure. Maximal recovery is observed after 16 hours anoxia. Desoxygenation must take place within 10 hours after irradiation to be effective (147).

Partial restoration can also be obtained by incubation of microorganism as *E. coli* at suboptimal temperatures after irradiation (148).

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# Deviations in nucleic acid metabolism induced by radiations


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(*Received* 24 *July* 1955)

It is with some diffidence that I would like to add a few remarks to the authoritative exposition given by Professor Hevesy. As he has pointed out the scope of radiobiology is now so vast that he has considered it advisable to restrict his choice of topics. We in turn only propose to comment on one or two of the issues he has raised.

Perhaps the most valuable part of Professor Hevesy's lecture has been an attempt to reconcile apparently conflicting experimental data and the interpretations put upon them. He has also emphasised that a very great step forward in our understanding of radiation lesions would be accomplished if we could explain the underlying cause of differential radiosensitivity. The great resistance of plants and bacteria compared to animals is fundamental but our understanding of the reason is negligible and is additionally complicated by the lack of biochemical knowledge of plants which might otherwise supplement the rather detailed knowledge of the mitotic interference in, for example, bean root tips (1) and *Tradescantia* sp. (2, 3). Some insight might however be gained from a knowledge of the differential radiosensitivity of animals cells and here it seems that Professor Hevesy's review has drawn together much of the information in this field. We might consider for a moment the part of his paper which is devoted to the disturbances manifest in cytoplasmic function. These include transient and cyclical variations in acid soluble phosphates of different tissues, particularly liver (4) and ascites tumor cells (5); glycogen accumulation (6) and alterations in basal metabolic rate (7). Although some of these have been observed very soon after whole body exposure to radiation there is nevertheless a possibility that they could be elicited as a secondary response of the intact animal. At a slightly later time cellular destruction may well lead to a release of nucleotides which are known to have a pharmacological action and might interfere with $^{32}P$ incorporation into ribonucleic acid (RNA) (8, 9). The utilisation of other cell fragments could account for glycogen accumulation.

Considerable attention has been paid to the role of endocrines in whole body radiation but evidence is still lacking which unequivocally establishes the participation of the pituitary-adrenal axis in any of the observed

lesions. The only experimental findings we have relevant to this showed that inhibition of $^{32}P$ incorporation into rat thymus nucleic acids was not prevented by adrenalectomy.

Early interference with enzyme activity has also been reported; an uncoupling of spleen oxidative phosphorylation (10, 11, 12) and an appearance of increased spleen adenosine triphosphatase. *In vitro* experiments which we have done (13) can be interpreted to suggest that the uncoupling is not due to a direct radiation effect on mitochondria and Dubois' recent observations (14) led him to conclude that the X-ray induced increase in phosphatase cannot be accounted for on the basis of direct damage to the spleen.

It is quite clear from Professor Hevesy's account that a tremendous effort has been directed to the elucidation of the disturbance in nucleic acid metabolism caused by radiation. Although this indeed appears to be an immediate consequence some caution is needed in the interpretation of experimental findings. Practically all the experiments have involved the use of either $^{32}P$ or a variety of $^{14}C$ precursors and the results with different tracers are not always in agreement. As Professor Hevesy has pointed out it is difficult to assess the extent to which the various metabolic pools are involved when radio-active tracers such as glycine are employed. In liver it has been shown (15) that the specific activity ratio RNA/DNA (deoxyribonucleic acid, DNA) using a number of different precursors, formate, 2-$^{14}$C-glycine, adenine and $^{32}P$ varies, with glycine and formate it is about 3 and with P and adenine about 15.

It is also clear that the interpretation of tracer experiments is radically affected if the pool increases or decreases in size, and this may have some bearing on the observed periodic fluctuations in the rate of $^{32}P$ incorporation into acid soluble phosphates after irradiation (4, 5).

Tracer experiments have been widely used to follow alterations in nucleic acid metabolism after exposure and again the experimental technique appears to modify the response. Total body radiation probably causes a greater inhibition in the organ than is obtained by direct irradiation with the rest of the body shielded. A further complication arises in the comparison of results given with young and old animals due to differing mitotic rates in particular organs. These two factors may well contribute to the quantitative differences which have been reported.

For example we were puzzled when we compared our results with those of Dr. Holmes on the degree of inhibition of $^{32}P$ uptake into DNA of regenerating rat liver (16). We obtained as much as 70 % inhibition but then found that there were two points of difference in technique. We used 100 g. rats and gave total body radiation whereas she used rats of 300-350 g. with local irradiation under amytal anaesthesia. In neither case was the RNA affected which also agrees with Payne, Kelly and Jones (15). There is however a variance in the RNA susceptibility which depends on the tissue studied. In intestine, bone marrow, spleen and thymus, RNA turnover is reduced and it is of interest to speculate on the reason for this difference.

It seems to us that effects on RNA are very closely connected with the situation in the nucleus and at present out ideas are crystallising round a primary action on DNA nucleoprotein, all other biochemical disorders resulting from this.

We have therefore been investigating the comparative radiation effects on $^{32}P$ incorporation into RNA and DNA in normal and regenerating liver, thymus and lymph node cultures. In all these tissues a measurable $^{32}P$ uptake was obtained and a greater inhibition was pro duced by radiation on DNA than RNA. This is especially evident in the liver where the effect on DNA contrasts sharply with a negligible inhibition both of cytoplasmic and nuclear RNA.

A similar result was obtained when lymph node cultures were employed. These cultures were kindly provided by Dr. Trowell and he assures us that although they survive in a normal histological state for at least 4 days only 1 in 20 000 lymphocytes are in mitosis (17). It has not yet been possible to determine whether the $^{32}P$ was incorporated both into lymphocyte DNA and also into that of the more radio-resistant reticulum cells, but the uptake was radio-sensitive.

At 2 h. there was a demonstrable inhibition of DNA $^{32}P$ uptake without much effect on RNA and histological examination gave the same pyknotic count as the controls; this was 6 %. When $^{32}P$ was added at 3 h. post radiation and the uptake again measured over a 2 h. period, 60 % inhibition of DNA uptake was associated with 30 % inhibition of RNA and 69 % of the cell nuclei were pyknotic.

TABLE I

*$^{32}P$ incorporation into nucleic acids of rat liver (at 2 hours after 1000 R X-radiation)*

| | Control RNA/DNA | Irradiated (as % control) | | |
|---|---|---|---|---|
| | | Cyto-plasmic RNA | Nuclear | |
| | | | RNA | DNA |
| Normal liver | 8.9 | 94 | 94 | 31 |
| | 5.2 | 85 | — | 97 |
| | 6.2 | 129 | — | 43 |
| Regenerating liver : | | | | |
| 28 h. | 1.05 | 81 | - - | 26 |
| 42 h. | — | — | 126 | 61 |
| 47 h. | 2.3 | 92 | 97 | 63 |

TABLE II

*$^{32}P$ incorporation into nucleic acids of rat lymph node cultures after 600 R X-radiation*

| | RNA | | DNA | |
|---|---|---|---|---|
| | 0-2 h. | 3-5 h. | 0-2 h. | 3-5 h. |
| Range | 89-126 | 46-78 | 46-79 | 33-41 |
| Mean and nr. of expts | 109 (6) | 69 (3) | 69 (6) | 38 (3) |

Data are expressed in % of controls.

Initially 10 μC $^{32}P$ were added/ml. incubation fluid which contained 35 μg. $^{31}P$/ml. After 2 h. incubation the average specific activity of the control DNA was 4.4 C.P.M./μg. P. If only dividing cells are incorporating $^{32}P$ into DNA the extent of the inhibition produced by radiation would only affect an insignificant proportion of the cell population, but in fact at 5 h. 69 % of the cells had pyknotic nuclei. This perhaps confirms Professor Hevesy's suggestion that interference in incorporation into DNA is but the observable consequence of so far undetectable disturbances.

There is still one further difficulty, we have to explain why normal liver and lymph nodes are so dissimilar in their reactions to radiation damage when we find that both suffer inhibition of $^{32}P$ incorporation. The reason for this difference may perhaps be due to the liver cell having more cytoplasm associated with its nucleus and a greater metabolic activity. This might act by producing a greater destruction of free radicals so that less damage is suffered by the nucleus, and in addition many cytoplasmic functions of the liver cell may be less dependent on the nucleus and therefore continue unperturbed.

When radiosensitive dividing cells are considered it is obvious that interference in DNA synthesis will have serious effects on subsequent mitoses and it is probable that the nuclear derangement will be communicated to the cytoplasm. At one time we wondered if the damage to the nucleus was different in dividing and non-dividing cells. We therefore studied the inhibition at 2 h. in $^{32}P$ incorporation into thymus nucleic acids of young rats over the range 25-3200 R.

The RNA was less affected than the DNA (figure 1).

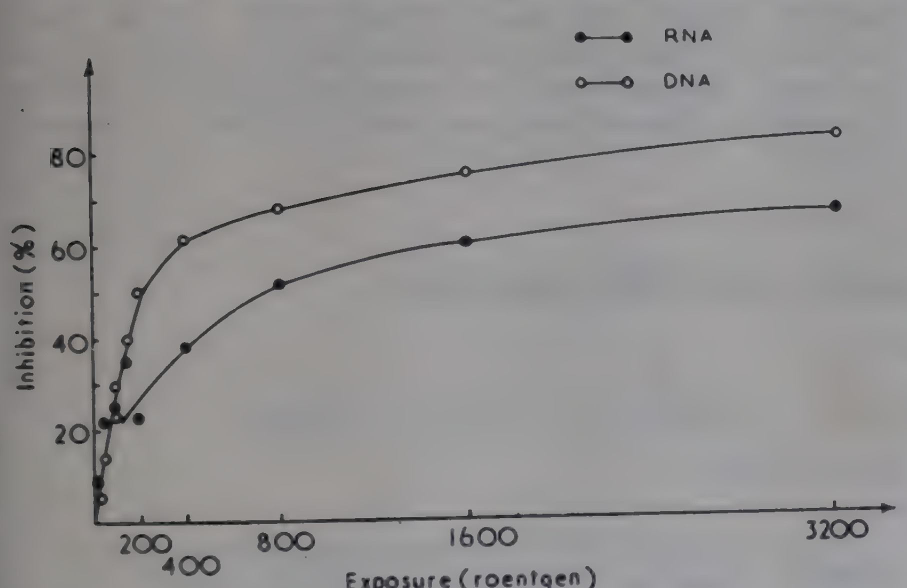


Fig. 1. — Inhibition of $^{32}P$ incorporation into rat thymus nucleic acids by X-radiation.

When the DNA data was plotted to give a probit log dose relationship a straight line was obtained (figure 2). This is similar to the one given by Trowell with lymph nodes (18) and provides no evidence for any peculiarity in the response of dividing cells. It seems possible therefore that there is only a single primary disturbance of the nucleus by radiation and that any further consequences depend on the nature of the cell itself.

Although it is easy to speculate on possible reasons for differential cell sensitivity it is difficult to design experiments which can with certainty establish a particular hypothesis, and it seems therefore desirable to continue work on the physico-chemical relationship of the nucleus and cytoplasm to see how these are disorganised by the stress of radiation.

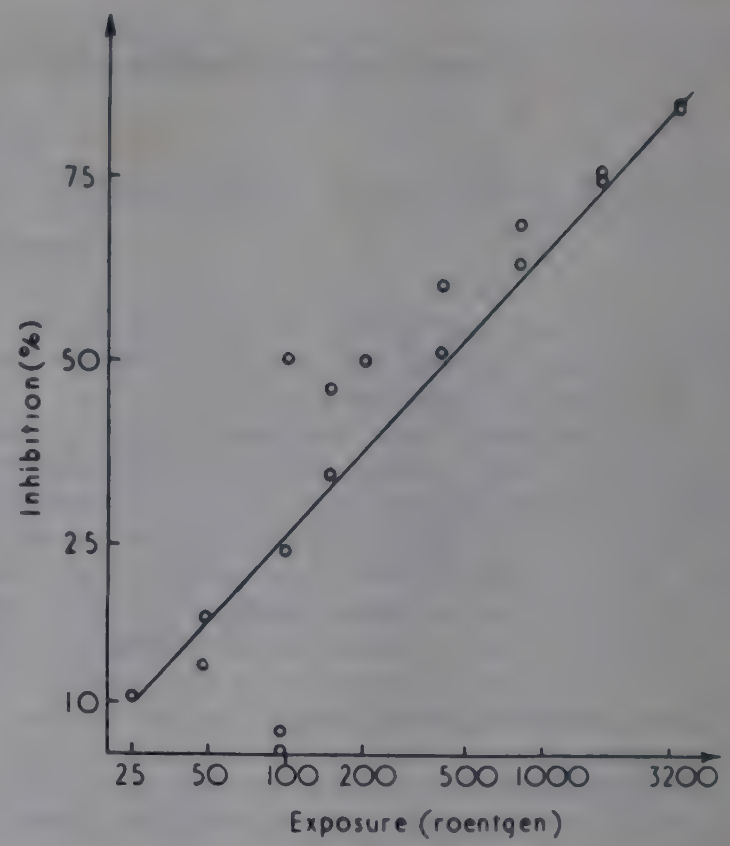


Fig. 2. — Incorporation of $^{32}P$ into rat thymus DNA after X-radiation. *Probit* dose : response curve.

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# A note on radiation effects on enzymes and macromolecules

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(*Received* 31 *July* 1955)

Professor Hevesy has shown in his lucid report how his brilliant tracer method has grown and been applied to an ever widening range of problems.

He has mentioned and discussed some biological radiation effects, not directly concerned with this tracer method, on which I should like to say a few words.

One point is the difficult question of how far, or whether at all, radiation effects on enzymes can play a part in the biological end-effect we see *in vivo*. I have an open mind on this question never having said more than that the action on enzymes is one possible mode of biological radiation effects which cannot be ruled out, and I consider this question as still undecided to-day, in spite of experiments which tend to show that even with heavy radiation doses many enzymes are not inactivated *in vivo*.

The conclusion that enzymes do not seem to play a part are based on two arguments, one being that in many cases no difference of total enzyme content could be found between irradiated and non-irradiated tissue and the other, as mentioned by Prof. Hevesy, that there would be too much protective protein present in the cell.

With regard to the first argument one has to bear in mind that an analysis could hardly detect a difference between irradiated and non-irradiated tissue because what is measured is the total amount of enzyme whereas only the minute functional part which is mobilized by the metabolic requirements of the cell is of significance when affected by radiation. Moreover the usual procedure of extracting enzymes from homogenized tissue yields variable amounts and may include non-enzymic activating or inhibiting substances.

With regard to the protective protein, one has to remember that in gel-like structures like protoplasm, the protein can be without effect, as shown in model experiments by Day and Stein. These authors irradiated methylene blue incorporated into a gelatine gel and did not find a protective effect of the gelatine micelles.

The other point I like to comment on concerns the conclusion that big molecules are more easily damaged by radiation than small ones. Professor Hevesy has quoted the splitting of the big molecule haemocyanin and the degradation of the long chain polymethyl-methacrylate molecule investigated by Alexander. Haemocyanin appears to be a particularly labile molecule which also dissociates by slight pH changes or small variations of the ionic strength of the solvent. With regard to polymethyl-methacrylate, Alexander found that 61 eV. of energy absorbed are able to break one main chain bond.

I should like to contrast these findings with results obtained on very much smaller molecules, namely : 32.5 eV. can oxidize 40 to 60 cysteine molecules to cystine, and the liberation of sulphur from thiourea, as we have recently found, can reach values of 90. These yields are due to chain reactions and are much greater than anything obtained with macromolecules and it seems as if examples of such chain reactions are found in increasing numbers.

# Irradiation, enzymes, hemoglobin and DNA synthesis

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(*Received* 15 *August* 1955)

In a recent book, written in collaboration with P. Alexander (1), I have insisted on the fact that in many instances, when biochemical changes were investigated soon, after irradiation (something like 30 min. or less) one often finds no decrease, but an increase of enzymatic activity, protein or RNA synthesis. In most cases it is proved that this increased activity is genuine, that it is not due to the destruction of an eventual inhibitor. We have reasons to believe that this increased activity is not the result of an increased synthesis of active molecules (enzymes or coenzymes) but to the fact that within the cell, the strategical position of enzymes and substrates is changed in such a way that either more substrate comes in contact with enzymes or that enzymes are 'set free' and meet substrates which normally are not available to them (see 1).

After this short early period of hyperactivity comes a deep depression in the synthesis of DNA, RNA and many proteins because the available stock of active substances and certain nuclear growth factors is rapidly exhausted and not rebuilt. Recent experiments in my laboratory on the giant unicellular alga, *Acetabularia mediterranea*, show that all the power for regeneration after irradiation is in the nucleus. The same damage is done to the cell if one irradiates the whole cell or the nucleus alone. Non nucleated parts of this organism (which for two or three months live and synthetise RNA as shown by Brachet *et al.*) are rapidly destroyed while a whole cell or a nucleus regenerates after 500 000 R. New instances of increased biochemical activity soon after irradiation are mentioned in the literature. We have found (with A. Nizet) an increased *in vitro* synthesis of hemoglobin

by dog's reticulocytes (2). Dubois has observed an increased nucleotidase activity of the rat's spleen after 400 R. This increased activity is inhibited if a protector (Patt's cystein or my cysteamine) has been injected before irradiation (3); thus, this biochemical trouble is probably an important step in the series of biochemical lesions which appear after a moderate does of ionizing radiations. Many enzymes seem to escape in blood and urine which are not normally present in these fluids or which, like DNAse are normally present in very small amounts only.

Just like my colleagues de Hevesy and Forssberg, whose contribution is fundamental in this field, I have been puzzled by the fact that many reports show an increase (or no significant change) in RNA synthesis and a simultaneous decrease in DNA synthesis. A few weeks ago, I received a personal communication (unfortunately without details) saying that with certain living material, very soon after irradiation, there is also a short period of increased DNA synthesis. If this observation is confirmed; one might put forward the general idea that all the main systems which regulate cellular growth and metabolisms are put out of order in the same way, but at different times, more or less rapidly; certain systems seem also more sensitive than others, *i.e.* are affected by smaller doses of ionizing radiations.

It seems to me obvious that biochemists interested in radiobiology should plan experiments in such a way that the duration of irradiation is short and the investigation undertaken as soon as posible after irradiation (Drs. de Hevesy and Forssberg have done so many times) or even during irradiation. One of the very important conclusions of the studies with chemical protectors is that the fundamental biochemical lesions are made during irradiation, since these protectors are absolutely ineffective if injected 20 to 30 sec. after the end of a 2 to 7 min. irradiation.

Last short remark : Dr. de Hevesy mentions in his report that Periston (a German low molecular weight polyvinylpyrrolidone) is active against radiation damage in mammals when administered after irradiation. We have repeated Burger and Lehman's experiments without success : Periston injected after irradiation (750 R) in various dosages does not modify the lethality curve in mice; given immediately before irradiation, it has a very slight protective effect.

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# Biochemical aspects of anaesthesia

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(*Received* 24 *May* 1955)

Narcotics, including anaesthetics, consist of a large variety of structural types such as hydrocarbons, alcohols, ethers, urethanes, sulphones, amides, ureides, barbiturates, etc. and it is evident that their common property of inducing narcosis in animals must depend partly on certain physicochemical characters which they have in common rather than upon the possession of any special chemical constitution.

The knowledge that brain and nerve tissues are rich in lipoidal substances led to the idea that lipoid solubility is in some way related to narcosis. Overton (1) and Meyer (2) independently suggested a parallelism between the affinity of an anaesthetic for lipoids and its anaesthetic effect. Using oil-water partition coefficients as measures of lipoid affinity, it was found that in many cases anaesthetic potency is related to the coefficient. On the other hand, it is known that many substances, more soluble in oil than in water, have no narcotic action, and that inorganic ions such as magnesium may induce anaesthesia and have little or no solubility in oil. Traube (3) advanced the theory that narcotics alter cell surface properties as a result of lowering of the surface tension of water, but not all narcotics lower the surface tension of water. Höber (4) suggested that permeability of the central nervous system is depressed by many anaesthetic agents and Winterstein (5) noted a decrease in permeability of muscle cells when treated with anaesthetics. Bancroft and Richter (6) claim that the ultimate action of a narcotic is the reversible coagulation of cell colloids and that, whatever the means by which this is secured (*e.g.* heat, cold, salts) such reversible coagulation will cause narcosis. Narcotic effects of nitrogen, and inert gases, argon, krypton and xenon, at high pressure have been attributed to lipoid solubility (7, 8) but Case and Haldane (9) doubt if this is the correct explanation. Ferguson (10) has reasoned that narcotic action may depend on a mechanism governed by the equilibrium existing between the concentration of the narcotic in the external phase and its concentration in a special sensitive phase. He concludes that equal narcotic effects are produced at equal thermodynamic activities in the sensitive phase and many calculations seem to support this conclusion (Brink and Posternak, 11). The rule, however, does not apply very rigorously, but even if it did, it would fail to show more than that the physical characteristics of the narcotic either determines its entry into a cell, or into a special phase of the cell, or its orientation at a specific surface. It gives no obvious clue to the mechanism of action of the narcotic. Butler (12) in 1950 concludes « that the study of the physical properties of anaesthetics is a method possessing distinctly limited possibilities for disclosing the actual mechanisms of narcosis... The nature of the physical effect is not clarified and the consequences of the primary action that lead to depression of cellular function remains to be described ».

Mullins (13) in a review concerned with the physical mechanisms affecting narcosis points out the significance of the movement of ions at membranes, and the functional importance of the sodium ion in nerve conduction. He concludes that when it becomes possible to determine what ions move, in a response to a stimulus « to produce membrane changes which permit movement of the sodium ion, it will be plausible to assign narcosis to a mechanism earlier in time than inward movement of the sodium ion ».

Such physical aspects are of undoubted importance in any consideration of the mechanisms of narcosis. But they present a limited view of the phenomena of narcosis and anaesthesia, just as a purely biochemical point of view presents a limited aspect, if the physical findings are not also taken into account. A combination of the results of both the physical and biochemical modes of investigation is necessary for that full description which must be the basis of any proposed mechanism of narcosis.

It has long been known that narcotics as a general rule inhibit enzymic and respiratory processus but until recently there has been reluctance to associate narcosis with a suppression of oxidative events. Verworn's classical theory (14) claimed that narcosis was a sort of asphyxia but Henderson (15) went so far as to state that there could be no relation between narcosis and oxidative events. Butler has also presented arguments against such an association. The reluctance in associating narcosis with oxidative events has been partly due to the fact that the quantities of narcotics required to induce narcosis in an animal are usually of a far smaller order than those required to inhibit enzymic reactions. Only recently has it been made quite clear that certain metabolic events in the nervous system are impeded by narcotics and anaesthetics at the low concentrations that bring about their pharmacological effects.

There has been ample demonstration of the existence of anoxia *in vivo* in the central nervous system during

the anaesthesia brought about by barbiturates and other narcotics. Himwich *et al.* (16) have found that, under pentothal, oxygen consumption of the cerebral cortex is decreased more than of the lower centres. With thiopental anaesthesia the average oxygen intake is lowered about 35 %. According to Shaw *et al.* (17) ether anaesthesia is associated with a decrease in the difference between the oxygen contents of arterial and venous bloods. Dameshek *et al.* (18) have shown that in the human subject under the influence of amytal there is a small but definite inhibition of oxygen uptake and dextrose utilisation by the brain. The depression of cerebral function by barbiturates indeed parallels the reduction of oxygen uptake. This fall in oxygen uptake *in vivo*, it is important to emphasize, however, may only be a reflection of the diminished cerebral activity obtaining under narcosis. It does not, itself, point to an interference with the respiratory activity of the nerve cells by a narcotic or anaesthetic.

Very many facts point to the profound influence of brain oxidations on its functional activity. Moreover, it has been clearly established that glucose is a principal substrate of the brain in the living animal (though, of course, it is not the only one) and that oxygen utilised by the brain *in vivo* is mainly concerned with combustion of glucose supplied by the blood. A deprivation of glucose from the brain has as dire physiological effects on the central nervous system as a deprivation of oxygen. It is known, however, that a variety of substances, other than those directly involved in a glucose breakdown are burned by the brain, as for example, glutamic acid (19) and a variety of amines (20). Doubtless the oxidation of these substances assumes greater importance when availability of glucose has for some reason diminished.

The physiological facts point to a very high degree of dependence of mental function on the maintenance of oxygen and glucose supply to the central nervous system. Any interference with the respiratory activity of the neurone, or groups of neurones in the nervous system, by the action of a drug would be expected to disturb its functional activity. The action of the drug, *i.e.* its biochemical effect, may be highly localised even if its distribution in the brain as a whole is uniform. The significant factor is the accessibility of the drug to the enzyme systems in the neurones affected. A large change, therefore, in the respiration, or other biochemical activity, of the entire central nervous system by biologically active concentrations of the drug need not necessarily be anticipated either *in vivo* or *in vitro*. A change of biochemical events at a particular centre in the central nervous system, paralysing the functional activity of this centre, and affecting, therefore, all branches of the central nervous system influenced by this centre, would not necessarily be accompanied by similar biochemical changes taking place in the nervous system as a whole as a direct consequence of the paralysis of the centre in question. It is for this reason that biochemical findings obtained in studies of the brain in the intact animal following drug administration must be interpreted with great caution. They may have little relation to the direct effect of the drug on the particular nervous centre affected.

### *Narcotics and respiration of the brain* in vitro

Narcotics have the power of inhibiting, at low concentrations, the respiration of isolated brain tissue. This conclusion applies also to local anaesthetics.

There is a parallelism among narcotics and anaesthetics of different chemical types between hypnotic activity and respiratory inhibition. Thus luminal, which is a more powerful narcotic than veronal, has a greater inhibitory effect on brain respiration *in vitro*. The same phenomenon holds with chloral and paraldehyde. This conclusion has been confirmed by various workers (*e.g.* Fuhrman and Field, 21 ; Watts, 22).

Results (30) obtained by using the brain cortex slice technique make it clear that a variety of narcotics at their narcotizing concentrations, produce inhibitions of respiration varying from 6 % to 32 %. When the respiration is stimulated by the addition of potassium ions, or by electrical means, these inhibitions are greatly enhanced (McIlwain, 23 ; Ghosh and Quastel, 24). The results are consistent with the view that the narcotics investigated produce significant inhibitions of the respiration of brain slices when they are present at concentration which in the organism produce deep narcosis.

Wilkins, Featherstone, Schwidde and Brotman (25) have shown in experiments, which combined a dog's brain biopsy method and the manometric technique, that the oxygen consumption *in vitro* of brain cortex, basal ganglion or hypothalamus, may be depressed to the extent of 70 % in the presence of 0.04 % pentobarbital. Moreover Schueller and Gross (26) have estimated the oxygen consumption of rat brain cortex slices in which the suspension medium consisted of whole blood drawn from a dog before and at given intervals after the administration of 36 mg./kg. nembutal. They found that highly significant inhibitions (25-33 %) of brain respiration are obtained using blood drawn 3, 15, 30 and 60 minutes after administration of the narcotic.

According to H. W. Elliott and Sutherland (28) the addition of pentobarbital (0.002 M) brings about a large inhibition of oxygen uptake of human cerebral cortex slices in presence of glucose, human brain cortex being a little more sensitive than rat brain cortex. The inhibitions recorded represent the effects of the narcotics on the respiration of the entire brain cortex of the animal. Local inhibition will be much higher if the narcotic is localized or specifically absorbed at particular centres or if its structure is such that it has a high affinity for enzymes located at certain sites. This seems likely as Swank and Cammermeyer (27) have obtained evidence that narcotics have differential effects on the neurones in brain.

### *Narcotics and specificity of oxidative inhibitions in the brain*

Narcotics and anaesthetics do not inhibit all oxidative processes to the same extent. The oxidations of glucose, lactate and pyruvate are most affected by the narcotics, while those of succinate and *p*-phenylenediamine are undisturbed. Watts (29) has pointed out that the more powerful local anaesthetics may impede succinate oxidation by isolated brain cortex. It is important to realize, however, that under certain conditions succinate oxid-

ation may not only involve direct oxidation to fumarate and malate, but further oxidation through oxalacetate and pyruvate. Hence inhibition of respiration caused by succinate oxidation may depend greatly on experimental conditions.

The high sensitivity to the narcotics of glucose oxidation in brain is a striking feature of narcotic action *in vitro*, and in view of the great importance of glucose oxidation in the functional activity of the central nervous system, and in, for example, so significant a physiological process as the synthesis in the brain of acetylcholine, this sensitivity must be taken into account in any consideration of the mechanism of narcotic action.

With brain, in contrast to such tissues as liver and kidney, carbohydrate breakdown is a dominant feature of metabolism and it is this fact which throws into prominence the specific inhibitory effects of narcotics in brain metabolism. It is to be borne in mind, however, that narcotics at quite low concentrations (Jowett and Quastel, 30) will also inhibit the oxidation of fatty acids and amino acids by isolated liver slices, and that narcotic inhibitions are not, therefore, completely restricted to oxidations of glucose and its breakdown products.

### *Reversibility of narcotic action* in vitro

The effects of narcotics such as the barbiturates, chloretone, or hyoscine, on the respiration of brain slices are reversible. High concentrations of narcotics, however, produce irreversible effects.

Analysis (30) of the kinetic data indicates that two effects of a narcotic on brain respiration *in vitro* takes place :

(*a*) Rapid attainment of an equilibrium between the narcotic and a constituent of the respiratory system. The inhibition of respiration is that to be expected from a simple mass action reaction, as observable with small concentrations of narcotics. This applies to narcotics such as urethane, chloral, chloretone, barbiturates, avertin (tribromoethyl alcohol) and magnesium ions.

(*b*) Relatively slow development of irreversible changes, leading to increased inhibitions of respiration that cannot be restored to normal by removal of the narcotic. This takes place with most narcotics such as barbiturates or chloretone at concentrations that effect inhibitions greater than 40 %. It may occur, however, at low concentrations with ether (31) and ethanol.

### *Respiration* in vitro *of brains of anaesthetized animals*

The brains of anaesthetized animals do not, usually, show a smaller rate of respiration *in vitro* than those of the normal. This is doubtless due to the diffusion of the narcotic from the brain slices into the surrounding medium thereby diminishing the narcotic concentration in the brain. For example, Wortis (32) found that the oxygen uptake of minced brains from rats injected with butyl-bromallyl barbiturate and sodium amytal was not diminished *in vitro* with glucose as substrate. K. A. C. Elliott and Henderson (33) found that slices of brains of rats and rabbits injected with pentobarbital showed no inhibition of oxygen uptake *in vitro*. Swift (34) gave lethal doses of barbital and avertin to rats but found that their brains *in vitro* showed no diminished rates of respiration from the normal. These negative results are to be expected if the narcotic effect *in vitro*, is, as has been shown may be the case, freely reversible. Taylor *et al.* (35), however, have found that when tridione and 3-ethyl-oxazolidinedione are injected into mice, the oxygen uptakes of the brains *in vitro* are inhibited 13 % as compared with the controls, while injection of 120 mg./kg. of phenobarbital results in 8 % inhibition. These authors found that the tridione content of brains of anaesthetized mice is approximately 0.0175 M, a concentration that produces 10 % inhibition of the normal oxygen uptake *in vitro*.

### *Effects of polassium ions*

Another phenomenon bearing upon the inhibitory effects of small concentrations of narcotics may now be considered. The steady state of the diminished respiration of brain slices brought about by small concentrations of narcotics, such as phenobarbital or chloretone, is found to be greatly dependent on the concentration of potassium ions in the medium (Jowett and Quastel, 30). With a concentration of K such as 0.0128 M, double that normally present in serum, a steady inhibition is quickly obtained by chloretone. At low concentrations of K, *e.g.* 0.002 M, however, the respiration drops quickly in the presence of narcotics to the level found with the higher K concentrations, remains at this steady state for a short period and then falls quickly so that eventually the inhibition found with low K is very much greater than with the high K concentrations. The concentration of K normally present in blood is sufficient to stabilize the inhibition due to the narcotic. When the temperature of the experiment, usually 39° C., is dropped to 29° C., the inhibitory effect of a narcotic such as chloretone no longer varies appreciably with the K. A steady inhibition is found whether the K is high or low.

The increasing inhibition at 39° C. due to low K, is possibly due to loss of K from the nerve cell at the low external concentrations (or to loss of some other cell constituent concerned with cell respiration) owing to irreversible changes in the cell that proceed more slowly when the temperature is lowered.

It is a well known fact that exposure of brain slices to relatively high concentrations of potassium ions *e.g.* 0.1 M results in a large increase in respiration in a glucose medium that may even exceed 100 %. This phenomenon is receiving greatly increased attention. It is now known (36) that the increased respiration due to the potassium is malonate sensitive and indicates a stimulation by potassium of the citric acid cycle operating in brain respiration.

Ghosh and Quastel (24) have found that the increased respiration of rat brain cortex *in vitro* brought about by the addition of potassium ions is greatly suppressed by the presence of pharmacologically active concentrations of barbiturates, chloretone and even ethanol. The increased sensitivity of the respiration of stimulated brain cortex to narcotics is due to a retarding effect of the narcotic on the specific phase of nerve respiration which is potassium-sensitive and which is concerned with

glucose (or pyruvate) oxidation. This phase is not prominent in a resting unstimulated nerve, the respiration of which is made up largely of processes unresponsive to potassium and insensitive to low concentrations of narcotics. This phase, however, becomes an important aspect of the respiration of stimulated nerve. It is known from the work of Lardy and Ziegler (37) that potassium ions are required for the catalysis of the interaction of pyruvate and adenosinetriphosphate; this reaction is among those of fundamental importance in the respiratory activity of the nerve cell.

The stimulating effect of the presence of 0.1 M potassium chloride on brain cortex respiration is seen not only with glucose as combustible substrate but also with fructose, sodium pyruvate and lactate. With all these substrates, the increased sensitivity of the respiration to the presence of a mixture of a narcotic is observable. In fact, in presence of these substrates and the narcotic little stimulating effect of added potassium ions can be seen.

With both succinate and glutamate as substrates, no stimulating effect of potassium on respiration is seen and neither is the narcotic inhibition in presence of potassium increased. It is clear, therefore that the presence of the high potassium concentration does not induce an increased sensitivity to narcotics of respiration as a whole. Only a specific phase of respiration, presumably that connected with the citric acid cycle of operation is largely affected.

It is of importance to point out that Buchel (38) has shown that removal of calcium ions from the medium has effects on brain cortex respiration and narcotic sensitivity similar to that due to the addition of excess potassium ions.

*Effects of demerol (1-methyl-4-phenylethylisonipecolate) and amidone (6-dimethylamine-4,4-diphenyl-3-heptanone)*

H. W. Elliott *et al.* (39) have examined the effects of demerol and amidone, which are held to resemble those of morphine, on brain oxidations. They found that these substances exert powerful inhibitory effects on the oxidation of lactic and pyruvic acids. This and other evidence led them to believe that the drugs inhibit the dehydrogenases involved.

*Effects of oxazolidinediones on brain respiration*

Tridione (3,3,5-trimethyloxazolidine-2,4-dione), which has been used extensively for the treatment of *petit mal* epilepsy, inhibits at low concentrations the oxidation of glucose by mouse brain cortex slices, and at higher concentrations the oxidations of pyruvate, lactate and glutamate (Taylor, Richards, Everett and Bertcher, 35). Succinate oxidation is the least affected. The inhibitory effect of tridione at low concentrations is reversible.

Propazone (5,5-di-*n*-propyloxazolidine-2,4-dione) according to Fuhrman and Field (40), inhibits the oxygen uptake of rat brain slices when glucose and pyruvate are substrates, but not when succinate and *p*-phenylenediamine are substrates. Epidon (5,5-diphenyloxazolidine-2,4-dione) was found to be a more potent inhibitor than propazone.

Among other anticonvulsants Wortis (41) has found that sodium bromide depresses oxygen consumption of brain slices *in vitro*.

Recent (unpublished) work by Geddes and Quastel has shown that the local anaesthetics, amethocaine, lignocaine, quotaine and nupercaine exert, at low concentrations, potent inhibitory effects on the respiratory activity of isolated rat brain cortex, these being markedly increased in presence of 0.1 M potassium ions. Thus, amethocaine (1 mM) brought about an inhibition of brain respiration in presence of glucose of 16 % that was increased to 45 % on addition of 0.1 M potassium ions. Lignocaine at 10 mM exerted little or no inhibition of rat brain cortex respiration in presence of glucose, but 40 % inhibition on addition of potassium. There was a striking parallelism between anaesthetic activity and respiratory inhibitory power in presence of 0.1 M potassium.

The significant role of potassium in stimulating a phase of nerve respiration that is highly narcotic and anaesthetic sensitive applies even to chlorpromazine, as new results by Lindan and Quastel using intact brain slices have shown.

*Steroids*

Steroids, which have anaesthetic potency, also affect rat brain (homogenate) respiration in presence of glucose but not in presence of succinate (Gordon and H. W. Elliott, 42; see also Eisenberg *et al.*, 43). The inhibitions parallel anaesthetic activities. There is an anomalous action of stilbestrol which is possibly accounted for by the special oxidative reactions undergone by this substance (Hochster and Quastel, 44).

It will be of importance to discover whether steroid inhibitions of brain respiration are affected by potassium ions. There is much interest at present in steroid-potassium relationships at cell membranes.

*Narcotics and dehydrogenases*

Low concentrations of narcotics that are highly inhibitory to aerobic oxidations in the brain have little or no inhibitory effect on anaerobic oxidations or on isolated dehydrogenases.

It seems that narcotics, at the low concentrations that are effective as inhibitors under aerobic conditions, exert their main effects on a part of the respiratory system that is inert or functionless under anaerobic conditions.

The inhibitions of respiration of intact brain tissue obtained by low concentrations of narcotics under aerobic conditions are, therefore, unlikely to be due to competition of the narcotics with substrates for their dehydrogenases, but are due to the affinity of the narcotics to special components in a complex of reactions that constitute the complete aerobic respiratory process of the nerve cell. This complex seems to be intimately associated with the citric acid cycle of respiratory operations.

*Narcotics and glycolysis*

The mechanism of anaerobic breakdown of glucose in brain involves the interplay of dehydrogenases that are also involved in the aerobic breakdown of the sugars. The absence of any effect of chloretone (45) on anaerobic glycolysis indicates that, if a narcotic-sensitive dehydrogenase system plays an important role in the aerobic breakdown of glucose, it is either absent from, or is

without influence in, the reactions involved in the anaerobic breakdown of glucose by brain. There is ample evidence, indeed, to show that narcotics increase aerobic glycolysis, by suppression of the Pasteur effect. Possibly this is the explanation for the observation of Edwards and Larrabee (46) that narcotics at low concentrations accelerate glucose consumption by excised rat superior cervical ganglia. This conclusion is in conformity with the observations of Greig (47), Rosenberg *et al.* (48), and Webb and K. A. C. Elliott (49) that in presence of narcotics there is an increased rate of breakdown of glucose though there is suppression of respiration.

It is useful at this stage to consider again the influence of potassium ions. The addition of potassium ions at 0.1 M brings about a suppression of anaerobic glycolysis of intact brain cortex amounting to over 40 % (50). Ghosh and Quastel, in unpublished work, have shown that this anaerobic potassium inhibition is entirely uninfluenced by the addition of narcotics such as chloretone or luminal.

If the presence of narcotics exercises an effect on membrane permeability so that the movements of potassium ions are affected, thus accounting possibly for the suppression of potassium stimulation of brain cortex respiration by narcotics, a similar suppression of potassium inhibition of brain cortex anaerobic glycolysis by narcotics would also be expected. This, however, is not the case. If a narcotic effect on nerve permeability occurs, this must be an aerobic phenomenon and be dependent, therefore, on oxidative events in the nerve cell.

### *Nerve cell energetics*

Although the evidence points to the participation of narcotics in the respiratory processes of the nerve cell, the problem still remains as to how an effect on a respiratory process, or some important aspect of this, can result in the phenomenon of narcosis. This is understandable only, it seems to the writer, in terms of cell energetics. A definite rate and direction of energy flow is essential for the normal functioning of the cell, and diminution or alteration of this, by a drug, may be expected to lead (if it is sufficient) to paralysis of the cell that is affected.

An essential aspect of respiration in the living cell, is the synthesis of adenosinetriphosphate (and other high energy catalysts of the cell) at the expense of the energy liberation by oxidations. On the basis of free energy release of 50 000-56 000 calories per g. atom of oxygen consumed and a free energy absorption of 12 000 calories for the conversion of ADP to ATP, the theoretical ratio of phosphate bonds formed to atoms of oxygen consumed (P : O ratio) is 4.2-4.7. Experimental ratios of 3.5-3.9 and even higher values are recorded (Hunter and Hixon, 51 ; Polis *et al.*, 52). It is known that phosphorylation is coupled with oxidation of reduced DPN (Lehninger and Smith, 53), reduced flavoprotein (Hummel and Lindberg, 54) and of participants in the cytochrome system. For the maintenance of nervous activity, a high level of high energy phosphate bonds is presumably necessary. Quastel, Tennenbaum and Wheatley (55) showed that synthesis of acetylcholine takes place in the brain at the expense of energy derived during the aerobic oxidation of glucose or of its breakdown products, lactate or pyruvate (though not of succinate in experiments *in vitro*). Nachmansohn and Machado (56) showed later that ATP is indispensable for acetylcholine synthesis and the process takes place anaerobically in brain homogenates and extracts when ATP is present. Evidently, during the aerobic oxidation of glucose, ATP enrichment occurs and this leads to acetylcholine synthesis. It is also known that the synthesis of acetylcholine depends on the integrity of the cozymase system present, this ensuring an optimal respiratory activity (Harpur and Quastel, 57).

In the presence of quantities of narcotics that suppress respiration, acetylcholine synthesis by brain is also suppressed. This was first shown using ether (58) as the narcotic. McLennan and K. A. C. Elliott (59) have shown considerable depression of acetylcholine synthesis by brain slices by various drugs, and Johnson and Quastel (60) have obtained similar results with narcotics. Ryman and Walsh (61) have also shown this using cocaine. There is a concomitant drop in rate of respiration but this need not necessarily be as large.

Narcotics or anaesthetics do not diminish the anaerobic synthesis of acetylcholine by ATP in presence of brain extracts (Quastel, 62 ; Johnson and Quastel, 60 ; Ryman and Walsh, 61). None of the known phosphorylations brought about by ATP (*e.g.* those involved in hexokinase activity and in glycolysis) are impeded by narcotics at low concentrations. At concentrations that impede respiration no inhibitory effect by narcotics or by cocaine was found on the acetylation of sulfanilamide by enzymes present in the liver.

When an acetylating system is linked with a respiratory system such as that present in the brain, the ATP necessary for the acetylation by these systems being formed by the energy of respiration, the presence of narcotics secures large inhibitory effects on the acetylation (Johnson and Quastel, 60). Typical results show that nembutal (0.5 mM) and chloretone (4 mM), at concentrations that do not affect anaerobic synthesis of acetyl sulfanilamide in presence of ATP, exert marked effects on the aerobic synthesis in the absence of added ATP. This applies also to an anaesthetic such as cocaine.

Grenell and Mendelson (82) have pointed out that narcotic concentrations of alcohols (ethyl, propyl, *iso*-propyl, amyl) do not interfere with the anaerobic acetylation of sulfanilamide and have no interfering effect with the activity of coenzyme A or with ATP utilization.

The addition of ATP to the aerobic system brings about a considerable alleviation of the inhibitory effect of the narcotic. The most obvious explanation of the inhibitory phenomenon, that takes place aerobically, is that the narcotics inhibit certain aspects of the respiratory process which are responsible for the oxidative synthesis of ATP. On this view, the major effect of a narcotic is not the suppression of respiration as a whole ; its effect, at low concentrations, is located on an aspect of the respiratory system which is responsible for the development of the high energy phosphate bonds of ATP. It follows from this that narcotics should affect a variety of metabolic processes dependent on the oxidative formation of ATP, a conclusion in harmony with the observations of McElroy (63).

It should be pointed out that Eiler and McEwan (64), have shown that pentobarbital inhibits the formation of high energy phosphate bonds by brain tissue proportionally to the same extent that it interferes with oxygen utilization. It is obvious that the hypothesis, that narcotics act by suppressing the rate of oxidative development of ATP in the nerve cell, has wide implications. Doubtless there are many aspects of metabolism in the nerve cell, equally important for its functional activity, which are controlled by the presence and rate of production of ATP.

The hypothesis that the main effect of a narcotic at low concentrations is to impede a reaction, or complex of reactions, in the sequence of events leading to complete oxidation of glucose, that is greatly concerned with ATP synthesis, makes it possible to understand why apparently large depressant effects on nerve function may take place with but little fall in the total respiration.

For example, it is known from the work of Larrabee *et al.* (65) that anaesthetics depress synaptic transmission in the excised superior cervical ganglion before a measurable decrease in oxygen consumption, as observed with the unstimulated preparation, is observed. The total oxygen consumption consists of the sum of the oxygen requirements for each of a variety of oxidative steps, and if only one of these is impeded at low narcotic concentrations the rates of oxygen uptake of the other reactions may not be affected so long as the enzymes involved are still saturated with their substrates. Yet, if the impeded step is largely concerned with the oxidative synthesis of ATP, all those activities of the nerve cell that are dependent on the presence of ATP will be affected.

Whether the synthesis of acetylcholine, or whether some other metabolic reaction dependent on the presence of ATP, is predominantly affected by the narcotic is at present unknown. Ryman and Walsh (61), from results of recent experiments with cocaine, conclude that the anaesthetic blocks the entry of acetyl-CoA into the citric acid cycle, a conclusion in harmony with our view that narcotics impede that aspect of respiration of the central nervous system that is concerned with the utilization of pyruvate by the operation of the citric acid cycle.

It is of interest to note recent results of Findlay, Strickland and Rossiter (66) who have shown that $^{32}P$ (as phosphate) is incorporated into isolated cat brain slices when this is respiring in presence of glucose or mannose. The $^{32}P$ is taken up into phosphoproteins and the 'residue' organic phosphorus. The glucose or mannose cannot be replaced by succinate or glutamate. This finding is to be contrasted with those of Abood and Gerard, and of Brody and Bain, that with particulate brain preparations both glutamate and succinate are able to support oxidative phosphorylation. The presence of respiratory inhibitors, such as cyanide or azide, or of narcotics, such as chloretone (4 mM) or nembutal (1 mM), exercises large inhibitions of $^{32}P$ uptake. It should be noted that Engelhardt and Lissovskaia had already shown that the presence of glucose under aerobic condition is necessary for $^{32}P$ incorporation into brain phosphoprotein.

Clearly, interference by narcotics, at low concentrations, with brain respiratory events causes widespread changes in phosphorylation processes.

Of particular interest are the recent observations of Bronk and Brink (67). These authors have shown that the rate of oxygen uptake by resting (frog) nerve is reduced 15 % by 0.002 M chloretone, but that the increment in the rate of oxygen uptake obtained with nerves carrying impulses at the rate of 50 impulses per second is decreased 50 % by the same quantity of narcotic. This result is consistent with the view that the narcotic impedes the aerobic synthesis of ATP whose presence may be required to secure the increased respiratory activity following electrical stimulation. It is well known that the presence of ATP is necessary to secure the optimal oxygen uptake of brain homogenates in presence of pyruvate. Bronk and Brink conclude that their results « indicate that chloretone inhibits an enzyme in a reaction located between the oxidative reaction directly affected by the impulses and the reaction using oxygen ». Our hypothesis conforms too with the conclusions of Fisher *et al.* (68) who have found, studying the effects of urethane on oxygen uptake and cell multiplication in yeast and in *Tetrahymena*, that the main action of the narcotic is confined to but one aspect of the total respiratory system, an aspect linked with the 'activity' metabolism whose magnitude is, doubtless, dependent, on the rate of aerobic synthesis of ATP.

When the respiration of rat and guinea pig cerebral tissue is stimulated electrically, it becomes much more sensitive to the action of barbiturates and chloral and mescaline (McIlwain, 69, 70), the inhibitory effect being reversed when the drug is removed. McIlwain concludes that the narcotic acts *in vivo* by inhibiting both energy yielding and energy consuming processes in the central nervous system.

McIlwain's interesting experiments (71) on the application of electrical impulses *in vitro* to animal, including human brain, tissue, have shown that both the respiration and aerobic glycolysis are markedly increased under these conditions. This applies to tissues immersed in glucose media, but not when succinate is the sole oxidisable substrate initially present.

It is an important fact to note that the activating effects of potassium ions (0.1 M) on respiration take place with the identical substrates (glucose, pyruvate, lactate) which permit responses to applied electrical impulses. With glutamate as substrate, according to McIlwain, human brain tissue maintains respiration which is increased on electrical stimulation, differing in this respect from many animal tissues. H. W. Elliott and Sutherland have pointed out glutamate oxidation is relatively vigorous with human brain tissue and is considerably inhibited by 2 mM dibucaine, the effect of the anesthetic being even greater with this amino acid than with glucose.

Just as potassium stimulated brain respiration is highly malonate sensitive (72) so is electrical stimulated respiration (Heald, 73). All evidence obtained so far, points to the similarity, if not identity, of biochemical effects secured by electric stimulation of intact brain with those obtained after application of potassium ions at 0.1 M concentration.

Electric stimulation brings about biochemical changes even in brain mitochondria, according to Abood and Gerard (74) but how far these are identical with those taking place in intact brain is still a matter for further investigation.

*Effects of narcotics on free and bound acetylcholine*

Turning once more to the consideration of acetylcholine in the brain, it has been known for some years that this ester is present in the brain in a bound form. Treatment of brain with a small quantity of a narcotic such as ether releases free acetylcholine from the bound form. This may occur at concentrations of the narcotic that do not markedly affect the respiration of the nerve cell. Since there is evidence that the conversion of bound into free acetylcholine is a rate limiting step in the synthesis of acetylcholine, it is evident that small concentrations of certain narcotics may in fact increase the rate of formation and liberation of acetylcholine. It is possible that this phenomenon is responsible, in some measure, for the excitatory effects of small doses of narcotics. It is known that acetylcholine has highly excitatory powers both on the neuromuscular junction (see Hunt and Kuffler (76) for a review of this subject) and on the magnitudes of brain potentials. Such a view of the mechanism of action of the apparently stimulant action of small concentrations of narcotics would be in harmony with what is known of the effects of narcotics on brain potentials (Tucci *et al.*, 77 ; Brazier and Finesinger, 78). Higher concentrations of narcotics, or narcotics acting for longer periods of time, will induce a depression of aerobic ATP formation, and an inability, therefore, of the nerve centres affected to maintain their normal function.

The inhibitive effects of narcotics and anaesthetics, at low concentration, on oxidative synthesis of ATP and, therefore, on the synthesis of the bound acetylcholine which presumably acts as store, to be drawn upon during the functional activity of the nerve cell, would account for a diminished rate of recovery of the cell to its normal condition. This impedance of recovery rate may well be the dominant factor in causing the paralysis of the nerve cell which is specifically affected by the narcotic or anaesthetic.

Richter and Crossland (79) have pointed out that the (total) acetylcholine content of rat brain, determined after rapid fixation by freezing with liquid air, varies with the physiological state of the animal. The level is significantly raised (40 %) above normal in animals examined during anaesthesia and sleep ; it is lowered in animals during emotional excitement, after electrical stimulation of the brain and during convulsions. It should be borne in mind that the amount of acetylcholine in the brain at any moment is the difference between the rates of synthesis and breakdown of acetylcholine. There rates may vary according to the physiological state of the animal and should be separately estimated to assess narcotic effects.

Rowsell (75) points out that electric impulses on brain slices *in vitro* release free acetylcholine from that in the bound condition and may bring about an accelerated synthesis as free acetylcholine *de novo*. Here again the results are precisely those found on application of potassium ions to brain cortex slices (Mann, Tennenbaum and Quastel, 80).

*Narcosis and uncoupling of phosphorylation*

A point of view, which is receiving much attention, is that narcotics at low concentrations will dissociate phosphorylation from oxidations without any apparent effect on the respiratory rate. It is well known that during respiration, organic phosphate esters are built up from inorganic phosphate in the tissues, and it has been shown that drugs such as dinitrophenol and gramicidin will inhibit this phosphate uptake without affecting the rate of respiration. It is claimed, for example by Brody and Bain (81) that barbiturates at low concentrations will also bring about the same phenomenon, an uncoupling of phosphorylation from oxidation. This point of view is similar to that already suggested, namely that narcotics impede the oxidative synthesis of ATP, with the exception that, on the former point of view, the action on oxygen uptake is relegated to a secondary effect.

The experimental basis for this point of view is that no drop in the rate of oxygen uptake can be observed at the low concentrations of narcotics that secure narcosis. This situation is changed, however, with the finding that narcotics effect considerable diminutions of brain cortex respiration activated by potassium or by electric stimulation. Moreover, as has already been pointed out, a small or negligible drop in respiration *i.e.* in total oxygen uptake, does not necessarily infer lack of action on an oxidative event. Although undeniably an 'uncoupling' of phosphorylation takes place, this is a process which is linked with an oxidative event upon which the narcotic must act to secure such 'uncoupling'. As has been stressed, the narcotics do not impede ATP synthesis when this takes place anaerobically.

It is interesting to note in this connection the observations of Grenell *et al.* (83) that chloretone at small concentrations inhibits respiration of brain homogenate in presence of pyruvate to a greater extent than the synthesis of ATP. Thus with 0.004 M chloretone there was 32 % inhibition of respiration and 12 % inhibition of ATP synthesis (see also Eiler and McEwen, 64, who found similar effects using pentobarbital). This is to be contrasted with the known inhibition of phosphate uptake by low concentrations of narcotics found when using mitochondrial preparations (Brody and Bain, 81).

A suggestion is made by Bain that narcotics, such as the barbiturates, act in a similar manner to dinitrophenol activating a 'latent' ATP-ase activity of brain, a direct hydrolytic attack on ATP not taking place but rather an increased rate of breakdown of some precursor of ATP. It will be interesting to observe whether such changes occur in potassium, or electrical, stimulated brain. It is noteworthy that dinitrophenol (1 mM) inhibits anaerobic acetylation of sulfanilamide caused by anaerobic pyruvate dismutation. Chloretone, even at 5 mM concentration, has no such effect (60).

The association of narcotic inhibitions with aerobic processes in the nervous system points to an interference with an underlying oxidative event. The inhibition of

oxidative phosphorylation *i.e.* 'uncoupling' leads not only to suppression of ATP formation but to the maintenance of ADP, which by acting as phosphate acceptor, may, under certain circumstances, stimulate oxygen consumption.

*Morphine*

It seems that with morphine an entirely different mechanism from that so far pictured is involved. It is known from the work of Quastel and Tennenbaum (84) that morphine competes with acetylcholine for receptor groups in the leech, the action being reversible and competitive. Similar phenomena occur with morphine derivates (heroin and codein) but not with apomorphine whose pharmacological action is quite different. The possibility arises that morphine and its allies act in the brain by competing with acetylcholine for those receptor groups whose combination with acetylcholine is essential for normal functioning of the neurone. Grundfest *et al.* (85) have recently put forward evidence that makes it seem likely that the curare group of substances owe their distinctive blocking action to competition with acetylcholine for a receptor protein. The protein is not choline esterase.

It is not proposed to discuss further, here, the mechanism of action of morphine and compounds of allied structure. It may, however, be pointed out that a narcotic or anaesthetic may have chemical features that enable it to act as a oxidative inhibitor or 'phosphorylation uncoupler' as well as an acetylcholine competitor at a receptor site.

*Future work*

So far as future work is concerned, it is evident that, from the biochemical point of view, much more information is required of the effects of narcotics, at the low concentrations that secure narcosis and anaesthesia, on the chemical changes associated with the energetics of the nerve cell, especially the stimulated nerve cell, and how far these effects may be correlated with physical changes, such as movements of cations at the cell membrane and electrical manifestations as shown in the electro-encephalogram. Moreover more information is needed on the relationship of narcotics to the physical structure of brain mitochondria, which are so sensitive to the action of narcotics and anaesthetics, and to the chemical events dependent on this structure. Only by a synthesis of all these results will we make real headway in the problem of narcotic mechanisms. A wealth of knowledge has accumulated concerning chemical structure and anaesthetic action and this obviously throws light on the factors securing access of the narcotic to the cell or the specific sites on which the narcotic acts. As biochemists, however, we wish to penetrate further than this and so far as I can see only further biochemical exploration will yield a satisfactory explanation of the mechanism of narcosis.

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## Introduction à la discussion : action des narcotiques sur le métabolisme et sur la fonction du système nerveux central


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(*Reçu le 4 septembre* 1955)

Le sujet que nous sommes appelés à traiter dépasse le domaine de la biochimie proprement dite. En effet, ce qu'on nous demande d'expliquer, c'est par quel mécanisme les agents extérieurs à l'organisme, les narcotiques, perturbent d'une manière réversible le fonctionnement du système nerveux central.

Aussi, pour plus de clarté, je soulignerai la distinction entre métabolisme du système nerveux central et activité nerveuse. S'il est évident qu'il n'y a pas de fonction sans métabolisme, une séquence causale ne signifie pas identité. L'activité fonctionnelle est liée au métabolisme de l'organe : la spécificité d'un organe réside d'abord dans sa fonction, et, dans une mesure beaucoup moindre, dans son métabolisme. Le métabolisme maintient l'activité fonctionnelle.

Il faut remarquer que la plupart des travaux biochimiques concernant le cerveau ne tiennent pas compte de sa topographie, de l'hétérogénéité des cellules nerveuses, et que ce qu'on mesure n'est que la moyenne statistique des unités douées d'activités fonctionnelles diverses. En outre, pour être dans le vrai, surtout dans une discussion sur la narcose, il faut, plus que jamais, tenir compte d'un

autre facteur très important, la structure. Claude Bernard a déjà dit que « l'anesthésique n'est pas un poison spécial du système nerveux », il agit sur les cellules « en arrêtant momentanément leur irritabilité ». Aussi faut-il prendre en considération les travaux de ceux qui, comme Overton-Meyer, Warburg-Fergusson, Eyring, Johnson, McElroy et autres, ont étudié l'aspect physico-chimique de la narcose. Mais n'oublions pas que notre tâche consiste à nous intéresser à l'aspect biochimique de la narcose chez les Mammifères supérieurs. Et, s'il est certain qu'une théorie générale de la narcose doit être valable dans ce cas en particulier, elle n'en sera pas moins insuffisante. Il me semble qu'on doit aussi tenir compte de l'action spécifique des narcotiques sur le système nerveux central. Et je crois utile d'attirer l'attention sur l'importance qu'il y aurait, sinon à combler le vide, du moins à jeter un pont entre ceux qui étudient l'aspect général et ceux qui étudient l'aspect biochimique, pharmacologique, physiologique ou électrophysiologique, en un mot l'aspect nerveux de la narcose.

Revenons au métabolisme, et posons la question : le métabolisme intermédiaire du cerveau diffère-t-il de celui des autres organes ? Le métabolisme intermédiaire du cerveau ne diffère pas du métabolisme intermédiaire des autres organes. Mais ce qui est plus spécifique pour le métabolisme du système nerveux central, c'est qu'il montre l'effet Pasteur, c'est sa teneur en acétylcholine, c'est sa plus grande dépendance de l'utilisation du glucose et son plus grand besoin d'oxygène ; le métabolisme du cerveau montre également une plus grande sensibilité aux vitamines $B_1$, à l'acide nicotinique ; n'oublions pas, enfin, que, la cellule nerveuse ne se divisant pas, il n'y a pas de croissance. L'énergie ainsi gagnée servira-t-elle à maintenir la cellule nerveuse dans un état instable qui est son état normal ?

Passant du métabolisme du cerveau à l'activité nerveuse, on peut dire que les processus fondamentaux qui caractérisent l'activité nerveuse supérieure sont des processus d'excitation et d'inhibition. Soulignons que le processus d'inhibition est également un processus actif.

Par quoi est caractérisée l'anesthésie chez les Mammifères supérieurs ? Elle est caractérisée par une excitabilité réduite, par la perte de conscience, par une diminution de l'activité électrique du cerveau, par un ralentissement de la circulation. Au niveau métabolique, elle est caractérisée par l'apparition de la glycolyse aérobie, l'inhibition de l'effet Pasteur, un déplacement de l'équilibre ionique entre le potassium et le sodium, une diminution du phosphore minéral, une teneur relativement élevée en adénosine triphosphate (ATP) et créatine-phosphate, une teneur faible en acide lactique, une augmentation de la teneur en acétylcholine et enfin une diminution de l'absorption d'oxygène. En ce qui concerne les composés phosphorylés riches en énergie, il s'agit donc de leur non utilisation plutôt que de leur épuisement.

Une question peut se poser : les narcotiques agissent-ils d'abord au niveau métabolique par la perturbation du métabolisme du glucose, ou agissent-ils d'abord au niveau de la fonction, de l'activité, en réduisant, au niveau des synapses, la transmission des impulsions dans les neurones du cerveau ? Je pense aux expériences de Larrabee et ses collègues : ils ont montré que les anesthésiques réduisent la transmission synaptique dans le ganglion cervical supérieur, avant la diminution de la consommation d'oxygène. Ou bien encore les narcotiques agissent-ils d'abord sur la structure, entraînant des répercussions à la fois sur le métabolisme et sur la fonction ? Etant moi-même biochimiste, je me rends compte qu'il nous arrive souvent de prendre le processus métabolique pour la fonction elle-même. Je suis certain que la discussion qui va s'ouvrir nous apportera quelque lumière, dans ce cas très précis, sur le lien entre le processus métabolique et la fonction.

En application de ce que nous venons de voir, l'étude biochimique de l'anesthésie comporterait donc : *a*) une étude de l'action des narcotiques sur le métabolisme intermédiaire du système nerveux, facteur non spécifique lié à la production et à l'utilisation de l'énergie ; *b*) une étude de l'influence des anesthésiques sur l'activité nerveuse, facteur spécifique, peut-être sur une substance dont l'action est spécifique, qui peut intervenir dans la transmission de l'influx nerveux, et dont la synthèse, le transport ou la dégradation peuvent être perturbés par les narcotiques.

Le rapport du Professeur Quastel a traité d'une manière exhaustive l'étude de l'influence des narcotiques sur le « facteur non spécifique », au niveau métabolique. L'étude du « facteur spécifique », celui qui intervient dans la fonction, est beaucoup plus difficile, étant donné que l'activité, c'est-à-dire l'influx nerveux, est peu connue. L'acétylcholine est-elle le « facteur spécifique » ou un des « facteurs spécifiques » ? C'est aux physiologistes qu'il appartient de répondre. Mais je puis vous dire que les expériences *in vitro*, concernant la synthèse ou la dégradation des deux formes de l'acétylcholine, libre ou combinée, en présence de certains barbituriques, ne nous ont pas donné d'indications.

Engelhardt et ses collègues attribuent aux phosphoprotéines un rôle spécifique dans l'activité cérébrale et considèrent le métabolisme intermédiaire comme un ensemble de processus préparatoires. Cette façon de voir correspond au schéma que je vous ai indiqué tout à l'heure, schéma dont je faisais déjà état en 1950. Nos connaissances sur la nature des phosphoprotéines sont insuffisantes, et Engelhardt et ses collègues, en utilisant le phosphore marqué, ont précisé les conditions de synthèse par le renouvellement du phosphore.

Le Professeur Quastel nous a donné dans son rapport un compte rendu détaillé de l'action des narcotiques sur les divers systèmes enzymatiques. Il ressort de cet exposé et de nos propres travaux, que les narcotiques n'ont pas une action directe sur les enzymes. Si on étudie l'oxydation du glucose avec des coupes de cerveau, on constate une diminution de l'absorption d'oxygène avec apparition simultanée de la glycolyse aérobie. Si on observe l'action des narcotiques sur les enzymes cristallisés, triosephosphate-déshydrogénase, aldolase, hexokinase, ou lacticodéshydrogénase, on s'aperçoit qu'aux mêmes concentrations que pour les coupes, ils n'ont aucun effet sur ces enzymes ; il faut une concentration dix à quinze fois plus forte pour obtenir une inhibition de 50 %. Ces résultats semblent indiquer que les narcotiques perturbent une chaîne de réactions dont le fonctionnement normal est lié à une structure. Ceci expliquerait qu'ils soient sans action sur les systèmes isolés.

La dénaturation réversible des protéines sous l'action des narcotiques, avancée par Johnson, Eyring, McElroy, suppose également un changement structural. Si la dénaturation signifie un gonflement, il s'ensuit qu'avec l'augmentation de volume provoquée par les narcotiques, les distances entre enzymes, coenzymes, transporteurs, fixés sur la structure, deviennent plus grandes, entraînant ainsi une perturbation dans les séquences métaboliques normales. Et le fait que les processus de phosphorylation et d'oxydation sont plus sensibles aux narcotiques que les processus glycolytiques est en accord avec cette hypothèse. En effet, le système de phosphorylation et d'oxydation est lié à une structure, alors que le système glycolytique peut être obtenu en solution.

Et si on étudie l'action des narcotiques sur le système de phosphorylation et d'oxydation seul, c'est-à-dire sur les mitochondries, on observe un découplage de phosphorylation, comme avec le dinitrophénol. On constate donc une diminution de la formation d'ATP, ce qui est en contradiction avec les résultats des expériences *in vivo*. C'est ici qu'il faut remarquer qu'on trouve des variations de sens opposé dans les expériences *in vivo* et *in vitro*, à la fois pour l'acétylcholine, le phosphore minéral, l'ATP, le créatinephosphate et l'acide lactique.

La teneur relativement élevée des composés riches en énergie, *in vivo*, peut s'expliquer non par une synthèse plus forte, mais par la non utilisation, comme nous l'avons dit plus haut. L'activité fonctionnelle, par exemple, l'excitabilité, diminue en présence des narcotiques : moins d'énergie est nécessaire pour le maintien de l'activité fonctionnelle réduite.

Vous avez pu remarquer que j'ai employé le terme de système de phosphorylation et d'oxydation et non celui de système oxydatif seul. Comme, avec les coupes de cerveau, le mécanisme déclenchant la glycolyse aérobie est beaucoup plus sensible aux narcotiques que ne l'est l'absorption d'oxygène, dans le cas des mitochondries, les phosphorylations sont également plus sensibles aux narcotiques que ne l'est l'absorption d'oxygène. Aussi je pense que la diminution de la respiration *in vivo*, s'il y en a une, n'est qu'un phénomène secondaire. Cette façon de voir permet d'expliquer l'apparente contradiction que représente l'inaction des narcotiques sur l'oxydation du succinate par les coupes de cerveau. En effet, les systèmes enzymatiques intervenant dans l'oxydation du succinate sont liés à la structure. Et nous avons dit plus haut que les narcotiques agissent sur la structure. Mais ce n'est pas du côté des processus oxydatifs, qui ne sont que secondaires à notre avis, qu'il faut chercher l'action principale des narcotiques au niveau du métabolisme, mais du côté du déplacement de l'activité ionique, et du côté de la phosphorylation.

Au niveau métabolique, on a trouvé récemment une réaction oxydative particulièrement sensible aux narcotiques : c'est l'absorption accrue d'oxygène due à l'ion K, au dinitrophénol, ou à l'excitation électrique.

Quastel a toujours pensé que les narcotiques agissent d'abord sur les processus oxydatifs, et, dans le fait que la respiration stimulée par le potassium est plus sensible à l'influence des narcotiques que ne l'est la respiration normale, il voit un argument de poids pour sa façon de considérer la narcose. Je me propose d'avancer une hypothèse différente de la sienne concernant le mécanisme de la narcose. Je pense que la diminution de la respiration, s'il y en a une *in vivo*, n'est qu'un phénomène secondaire, et je vois l'action primaire des narcotiques au niveau métabolique, qui est une conséquence du changement structural et dont je vous ai entretenus plus haut, dans la suppression de l'effet Pasteur, dans l'action glycolysante, démontrée par nous. En quoi consiste cette action *in vitro* ? Elle consiste en une consommation plus forte du glucose (environ 50 %), une formation plus importante d'acide lactique (environ 180 %), un changement de perméabilité, une sortie du potassium de la cellule et une entrée de sodium. Le mécanisme déclenchant la glycolyse aérobie est beaucoup plus sensible aux narcotiques que la respiration : quand la respiration diminue de 30 % par exemple, la glycolyse aérobie augmente de 300 %. C'est pour cette raison que je pense qu'une éventuelle diminution de la respiration est due aux réactions secondaires. En présence des narcotiques, nous avons à la fois la respiration et la glycolyse aérobie avec ses conséquences sur le rendement énergétique et sur le changement de perméabilité de la membrane. Si la présence du potassium à l'intérieur de la cellule est nécessaire pour le maintien de l'excitabilité de la cellule nerveuse, l'appauvrissement de la cellule en ion potassium, provoquée par l'action des narcotiques sur la perméabilité de la membrane, entraîne une diminution de l'excitabilité, et c'est ce qu'on observe *in vivo*.

Notre explication de l'effet du potassium consiste donc en ceci : le mécanisme qui déclenche la glycolyse aérobie est particulièrement sensible à l'influence des narcotiques, probablement en raison de leur action sur la structure. Le changement ainsi provoqué dans la cellule se traduit par un changement de perméabilité, par le déplacement de l'équilibre ionique, par une sortie importante de l'ion potassium de la cellule et l'entrée de l'ion sodium, ceci entraînant une diminution de l'excitabilité. La diminution de la respiration, s'il y en a une *in vivo*, n'est qu'un phénomène secondaire.

En résumé, on peut dire que la cellule nerveuse se trouve dans un état particulièrement instable. C'est son état normal à l'état de veille. Elle a besoin d'une grande dépense d'énergie pour maintenir cette structure très complexe. Tout changement provoqué dans une telle structure est un processus actif ; même son inhibition est active, requiert de l'énergie. Il est évident qu'une telle structure soit sensible à l'influence des narcotiques dont l'action est d'abord physique. Ce changement de structure provoque la suppression de l'effet Pasteur, un changement de perméabilité, un déplacement de l'équilibre ionique entre potassium et sodium. La diminution des processus oxydatifs, s'il y en a une *in vivo*, n'est qu'un phénomène secondaire. Le déplacement de l'équilibre ionique peut expliquer la diminution de l'excitabilité de la cellule nerveuse.

Je sais bien que la diminution de l'excitabilité de la cellule nerveuse n'est qu'un aspect de la narcose ; loin de moi de prétendre que les quelques faits cités plus haut et l'interprétation que j'en ai donnée expliquent la narcose.

Au cours de cet exposé, j'ai insisté sur les trois niveaux auxquels doit se faire l'étude de la narcose : *a*) au niveau de la structure ; *b*) au niveau du métabolisme ; *c*) au

niveau de la fonction. Nous possédons des données nombreuses concernant l'action des narcotiques sur la structure, sur le métabolisme et sur la fonction, étudiés séparément. La tâche des recherches futures doit être d'expliquer en termes de mécanisme les changements que provoque une perturbation due aux narcotiques sur la structure au niveau métabolique, d'expliquer en termes de mécanisme les changements que provoque une perturbation de la structure et du métabolisme au niveau de la fonction; il s'agit en somme d'établir une séquence causale entre les trois niveaux. Nous sommes déjà en possession d'un certain nombre de données concernant les liens entre structure et métabolisme; mais il reste beaucoup à étudier concernant les relations entre le métabolisme du cerveau et l'activité cérébrale, avec ou sans narcotique.

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## Brain acetylcholine during insulin hypoglycaemia, anoxia and anaesthesia

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(*Received* 9 *September* 1955)

Professor Quastel suggested in his survey that the susceptibility of glucose oxidation to narcotics *in vitro* might indicate an impaired synthesis of bound acetylcholine in the intact anaesthetised animal. He further suggested that this might come about through an impairment of the oxidative synthesis of ATP. I wish to confine my remarks to the effects of hypoglycaemia, hypoxia and anaesthesia on the acetylcholine content of brain, but I must first point out, as has already been mentioned by Dr. Rosenberg, that *in vivo* experiments have shown that the ATP content of brain actually increases during anaesthesia (1). I have always, indeed, been impressed with the fact that the ATP stores of the brain remain intact even in such extremes as fairly severe hypoxia (2). Only during convulsions (3, 4, 5) and hypoglycaemia (6) is there any obvious depletion of these stores.

There is certainly evidence that the synthesis of acetylcholine *in vivo* is dependent on glucose oxidation. Thus we have recently shown that the acetylcholine content of the brains of young rats, frozen *in toto* in liquid air, is markedly depressed during insulin hypoglycaemia (7). Our results emphasise that the reduction in brain acetylcholine occurs not merely secondarily, as a result of convulsive activity (which in non-hypoglycaemic animals certainly does cause a depletion of the acetylcholine stores in the brain (8, 9)) but during the stage of depression when, in the absence of impaired acetylcholine synthesis, one might reasonably have expected the total acetylcholine of brain to rise (8). We have tentatively interpreted our results as indicating inhibition of acetylcholine synthesis during hypoglycaemia.

Evidence for a similar effect of hypoxia is not obtainable in so direct a manner, for the exposure of an animal to an atmosphere deficient in oxygen leads to changes in its cerebral activity which might well, of themselves, affect its brain acetylcholine. We have, however, obtained indirect evidence for an inhibition of acetylcholine synthesis during hypoxia as follows.

Adult rabbits were tracheotomized and craneotomized and electrodes, for electroencephalogram (EEG) recording, inserted into a remaining bridge of bone. Some time after the operation, while the animals were still deeply anaesthetised, liquid air was poured over the exposed brain, the frozen cortical layers chipped off and the acetylcholine content of the cortex determined in extracts of the frozen tissue. In a series of six experiments, the mean value obtained for the cortical acetylcholine was 2.1 μg./g. Another series of rabbits was treated in a similar fashion except that, after operation, the animals were given an intraperitoneal injection of leptazol. This caused the appearance of convulsive spikes in the EEG, which were allowed to continue for 15 minutes before applying liquid air. The effect of the convulsive activity was to cause a marked reduction in the cortical acetylcholine content to 1.3 μg./g. (6 experiments). In a third group of six rabbits, the same procedure was adopted but after only five minutes of convulsive activity the animals were given nitrogen to breathe, with artificial respiration when necessary. This caused a rapid and very marked reduction of cortical activity. Fifteen minutes after the appearance of the initial convulsives spikes the cortex was frozen. It was found that, in spite of the reduced activity of the cortex during the major part of the experimental period, its acetylcholine content was now only 0.82 μg./g. The most reasonable explanation of these results is that the period of anoxia caused impaired acetylcholine synthesis. The readmission of oxygen to the inspired air following the period of complete anoxia caused a rapid return of the convulsive spikes and of the acetylcholine level (to 2.1 μg./g.).

While it does seem reasonable, therefore, to suggest that acetylcholine synthesis might be impaired when supplies of either glucose or oxygen are limited, there is no evidence of any such inhibition of acetylcholine synthesis during anaesthesia. It has been known for some time that the acetylcholine content of brain increases during anaesthesia (10, 8, 11) but such a rise is not, of course, necessarily incompatible with a reduced acetylcholine synthesis. Nevertheless, whether synthesis is impaired or not, the actual amount of (presumably available) acetylcholine increases and the brain is not prevented from acquiring a considerable 'store' of acetylcholine. We have evidence, indeed, that during anaesthesia the brain becomes 'saturated' with acetylcholine. Table I shows the fractional increase in the acetylcholine content of the brains of young rats, subject to deep anaesthesia with a variety of anaesthetics, for varying periods of time.

TABLE I

*Increase in the acetylcholine content of brain during anaesthesia*

| | Percentage increase |
|---|---|
| Chloroform | 61 ± 10 (7) |
| ' Dial ' | 58 ± 9 (8) |
| Chloralose | 54 ± 9 (10) |
| Ether | 62 ± 8 (13) |
| Ethyl chloride | 50 ± 8 (5) |
| Pentobarbitone | 47 ± 8 (7) |
| Thiopentone | 59 ± 13 (7) |

Each anaesthetised animal was paired with an unanaesthetised litter mate; the numbers in brackets indicate the number of pairs of animals used.

It is clear that all the anaesthetics tested had a very similar quantitative effect on the acetylcholine content of the brain; however long the anaesthetic was prolonged (up to 2 hours) there was no further increase beyond the 50 % noted in the table. These facts can best be interpreted on the assumption that, while the brain of the unanaesthetised animal does not hold its full complement of acetylcholine, it does become fully 'saturated' during anaesthesia. Such an assumption would explain the steady, uniform acetylcholine content of brain under different anaesthetics, as well as the fact that we have not been able, by any treatment, to raise the level of the total acetylcholine in brain beyond that found during deep anaesthesia. It is of interest, in this connection, that while there is a well-established difference between the acetylcholine content of unanaesthetised young and adult rats, this difference is abolished in deep anaesthesia.

Apparently, then, the capacity of the brain to store acetylcholine, as measured by the level found in deep anaesthesia, reaches its maximum before the acetylcholine content of the brain of the unanaesthetised animal reaches that characteristic of the adult. If the rate of loss of acetylcholine, due to nervous activity, is such that, in the unanaesthetised animal, synthetic processes are insufficient to maintain a maximum acetylcholine level, the differences between young and adult animals might be attributed to a relatively greater cerebral activity in the former group.

Pentobarbitone anaesthesia does not cause quite as large an increment of brain acetylcholine as does ether in the young animal; this difference between the effects of the two anaesthetics is not evident in the adult animal. This point was investigated in more detail by studying the effects of both anaesthetics on the acetylcholine content of four large areas of the young and adult brain. The results are summarized in tables II and III.

It can be seen that the smaller effect of pentobarbitone as compared with ether in the young animal is due entirely to its causing a smaller increase in the acetylcholine content of the cerebral hemispheres. In the adult animal, on the other hand, there is no difference between the effects of the two anaesthetics on the cerebral hemispheres, but pentobarbitone now has a smaller effect than ether on the medulla and pons. The medulla and pons form so small a part of the total brain mass that the reduced effect of pentobarbitone on this region does not make itself evident in the figures for the total brain acetylcholine in the adult. Now, the acetylcholine content of the cerebral hemisphere of the young animals is relatively low; it increases in the adult animal, while that of the medulla and pons falls. In other words, those parts of the brain with a relatively low resting acetylcholine content are the ones which do not acquire as high a complement during pentobarbitone anaesthesia as they do under ether. Welsh has suggested (12) that areas of the brain of low acetylcholine content are particularly susceptible to hypoxia and, since it is well known that there is usually a tendency to hypoxia during barbiturate anaesthesia, it might well be that the low values for acetylcholine in the cerebral hemispheres of the young rat, and in the medulla and pons of the adult, are due to impaired acetylcholine synthesis consequent upon a hypoxia. Thus, the intervention of hypoxia during anaesthesia can apparently retard the accumulation of acetylcholine by susceptible areas of the brain. It should, perhaps, be added that if animals are deliberately made hypoxic during anaesthesia the effect of pentobarbitone on the cerebral hemispheres and medulla and pons becomes more marked.

Since pentobarbitone tends to oppose the changes, at any rate of acetylcholine content, which appear to be

TABLE II

*Distribution of acetylcholine in different parts of the brain of young rats (25-30 g.) during ether and pentobarbitone anaesthesia*

| | Cerebral hemispheres (µg./g.) | Upper brain stem (µg./g.) | Cerebellum (µg./g.) | Medulla and pons (µg./g.) |
|---|---|---|---|---|
| Ether anaesthesia : | | | | |
| Normal | 1.9 ± 0.03 | 3.0 ± 0.03 | 0.35 ± 0.03 | 2.5 ± 0.38 |
| Anaesthesia | 3.7 ± 0.23 | 4.1 ± 0.13 | 0.72 ± 0.21 | 3.6 ± 0.30 |
| Pentobarbitone anaesthesia : | | | | |
| Normal | 1.8 ± 0.03 | 2.9 ± 0.07 | 0.35 ± 0.02 | 2.6 ± 0.41 |
| Anaesthesia | 3.0 ± 0.22 | 4.1 ± 0.20 | 0.72 ± 0.05 | 3.4 ± 0.50 |

Each figure represents the means and standard errors of the means of four observations, each of which was made on the pooled brains of two rats.

TABLE III

*Distribution of acetylcholine in different parts of the brain of adult rats (200-250 g.) during ether and pentobarbitone anaesthesia*

| | Cerebral hemispheres (μg./g.) | Upper brain stem (μg./g.) | Cerebellum (μg./g.) | Medulla and pons (μg./g.) |
|---|---|---|---|---|
| Ether anaesthesia : | | | | |
| Normal | 2.8 ± 0.25 | 3.6 ± 0.17 | 0.44 ± 0.07 | 1.9 ± 0.20 |
| Anaesthesia | 3.8 ± 0.26 | 4.4 ± 0.09 | 0.63 ± 0.09 | 3.1 ± 0.24 |
| Pentobarbitone anaesthesia : | | | | |
| Normal | 2.8 ± 0.25 | 3.6 ± 0.17 | 0.44 ± 0.07 | 1.9 ± 0.20 |
| Anaesthesia | 4.0 ± 0.24 | 4.1 ± 0.20 | 0.60 ± 0.09 | 2.4 ± 0.10 |

Each figure represents the means and standard errors of the means of four observations, each of which was made on the pooled brains of two rats.

characteristic of the state of anaesthesia, it is questionable whether this drug, so often employed in anaesthetic studies, is a satisfactory agent for throwing light on the fundamental mechanisms of narcosis.

Anaesthesia is a synaptic phenomenon : transmission, for instance, through a sympathetic ganglion, is blocked by concentrations of anaesthetics too low to impair conduction in the fibres (13) and it is worth noting that a large group of substances (including, significantly enough, the local anaesthetics) can block synaptic transmission at this site by preventing the release of acetylcholine. A similar mechanism might well operate at central synapses. It is to be remembered that, while it is probable that acetylcholine does play the role of synaptic transmitter at some sites in the central nervous system, it is equally probable that it is by no means the only transmitter agent. A theory of anaesthesia which is too much concerned with attempting to implicate those biochemical systems responsible for acetylcholine synthesis is neglecting the probably important role played by these other neurohumours. If, however, we direct our attention to those mechanisms whereby, in anaesthesia, the release (rather than the synthesis) of a transmitter substance might be inhibited, the difficulty of having to explain the action of anaesthetics as impairing the synthesis of multiple transmitter substances is overcome. The results presented here are, of course, compatible with such an impaired release of acetylcholine. They are certainly not compatible with the hypothesis that anaesthesia can be causally related to impaired glucose metabolism or failure of ATP synthesis, since the changes in acetylcholine content produced by such conditions are the opposite of those occurring during anaesthesia.

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## Remarks concerning the mechanisms of narcosis and anesthesia

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(*Received* 24 *October* 1955)

It has been a great pleasure to read Dr. Quastel's paper as well as to hear that of Dr. Rosenberg. For several years we have hoped to find our point of view in agreement with that of Dr. Quastel, and it was pleasant indeed to find some thoughts in his paper about which we feel the same. None the less, there are many points in his thesis about which I should wish further clarification. I cannot say that we have been impressed by so-called evidence in favor of any part of any one series of steps in intermediary metabolism being the crucial point of action of narcotics and anesthetics, whether or not it be reflected in the test of oxygen consumption, or if it appears to interrupt any one series of cyclic reactions.

The question of the concentration of the narcotic is

most vital. A good deal of the data on which Dr. Quastel's views are based was obtained from experiments in which unnecessarily high, unphysiological concentrations of the drug were used. Recent results of our experiments as well as of those in other laboratories demonstrate that many narcotics and anesthetics in physiological, narcotic concentrations do not inhibit respiration, *i.e.*, may have slight or even no effect on the oxygen uptake of the brain. Neither chloretone nor sodium pentobarbital depress the $QO_2$ when they are present in physiologically effective concentrations. Indeed, there are indications that low enough concentrations may even stimulate. Associated with this is the evidence demonstrating that with the lower concentrations of narcotizing agents there is either a slight increase or no shift in the steady state level of ATP. The net synthesis of ATP in no case appears to be significantly depressed by the narcotic substances in reasonable concentration. It is possible that at low, physiological concentrations of the narcotics, oxidative phosphorylation as well as a pathway of ATP utilization are partially inhibited leading to an unchanged steady state level of ATP. If it is true that oxidative phosphorylation is inhibited by the low narcotic concentrations while the overall ATP concentration is maintained at a normal level, it suggests the possibility, if ATP level is of importance in narcosis, that the function of a particular cellular structure is important insofar as narcotic action is concerned.

It is most vital (and we are heartily in agreement with Dr. Rosenberg in this point) that we consider carefully the role of activity levels in these problems. Sensitivity of a system changes with change in activity. Further, any part of the nervous system being considered must be thought about in terms of its state of activity at the time analyses are being made. Recent work in our laboratories and those of Dr. McElroy shows quite clearly the direct relationship between the degree of activity of nerve cells and the utilization of ATP. Some of the experimental data are shown in table I.

TABLE I

*ATP of rat cerebral cortex*

| Experimental group | ATP (µg./g. wet weight) | % Change from control |
|---|---|---|
| Unanesthetized (control) . . | 75.15 | |
| Anesthetized (nembutal) . . | 184.70 | + 145 % |
| Metrazol (unanesthetized) : | | |
| Early = clonic phase . . . | 41.70 | — 44 % |
| Late = tonic phase . . . | 22.50 | — 69 % |

Lack of time will not permit detailed discussion, but we must recognize that any theory serving to explain the action of narcotics and anesthetics must deal with structures and processes in the membranes of cells and cell particulates, as well as with structures and processes within the cell and/or its components. We wish to suggest an hypothesis (to be published at length elsewhere) which deals with :

(*a*) the effects of narcotic and anesthetic molecules on cellular, nuclear, mitochondrial and other membranes; and,

(*b*) effects of these substances on enzyme systems attached to these membranes, which are concerned with oxidative reactions leading to the generation of phosphate bond energy, general group transfer and exchange reactions, and cellular permeability with the unequal distribution of ions to the various metabolic reactions.

It is important to remember that phosphate bond energy represents only one type of biological bond energy. In suggesting that phosphate bond energy may be important in narcotic action, it is assumed that such energy is in equilibrium with and reflects the level of other forms of bond energy. Recent studies on group transfer have clearly shown that phosphate is not necessarily an obligatory component, and in fact, may be separated structurally from those states which could immediately equilibrate these other energy-rich groups with phosphate bond energy. Thus narcotics may interfere, in time, with phosphorylation processes, but they may be doing so by affecting group transfer processes in which the energy of the group would be lost as heat. The speed would depend upon structural and other considerations.

Evidence would suggest that in narcosis and anesthesia there is inhibition of transfer, alteration of structure, greatly decreased esterification of inorganic phosphate and thus an inadequate synthesis and utilization of the external adenylic system to maintain normal structure. This type of disturbance could easily explain the differences in action of different narcotic substances as well as the induced states of early stimulation followed by depression of functional activity.

# The determination of steroids in blood and urine

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(*Received* 21 *May* 1955)

## *Introduction*

It must be emphasized at the outset of this symposium that the problem of the analysis of blood and urine for steroid hormones and metabolically related steroids is not only beset with considerable technical difficulties but is also a very complex one because of the multiplicity of different steroids which have to be taken into account in considering each of the steroid hormone-secreting glands. This multiplicity arises in two ways :

— Firstly, each of these glands elaborates, and in some cases certainly secretes, not a single hormone, but a number of different physiologically active steroids; while in some instances there is reason to believe that 'physiologically inactive' (*) steroids are also elaborated and perhaps secreted.

— Secondly, the metabolism of the steroid hormones in the tissues after they are secreted is complex, each hormone giving rise to a number of different metabolic products which are excreted in the urine and which presumably are also present in the blood.

In the time allotted it will not be possible to consider the complexities involved in the biogenesis and metabolism of the steroid hormones in any detail, but a brief reference to them must be made in order to provide the background for our discussion.

*The Graafian follicle.* — It is probable (but not definitely proved) that the human Graafian follicle secretes two different œstrogenic hormones, œstradiol-17β and œstrone. These two œstrogens are metabolically interconvertible to some extent in the tissues, and both are excreted in the urine. In the tissues these two œstrogens also undergo partial conversion to the closely related œstriol, and also, probably to the isomeric 16-*epi*œstriol. Both of these œstrogen metabolites are excreted in the urine.

*The* corpus luteum. — The *corpus luteum* secretes not only progesterone, but also œstrogens; and there are some reasons to suppose that the latter may include œstriol as well as œstradiol-17β and œstrone.

After it is secreted progesterone undergoes metabolic reduction in the tissues. Since the reduction of the αβ unsaturated ketonic group in ring A and of the ketonic group at C-20 involves the formation of three new centres of asymmetry the total number of partial and complete reduction products which might be formed from progesterone is large. In fact no fewer than nine of the theoretically possible reduction products of progesterone have been isolated from human urine, although only four of these have been definitely proved to be formed from progesterone metabolically.

*The placenta.* — In man the placenta assumes the endocrine functions of the ovary during pregnancy after about the third month, and secretes progesterone and œstrogens. Oestradiol-17β, œstrone and œstriol have been isolated from human placental tissue, and there are grounds for believing that all three may be secreted and can therefore be regarded as placental hormones : progesterone has also been isolated from this source. There is also some evidence for the presence in placental tissue of androgens and of adrenocortical hormones.

*The testis.* — While the principal hormone of the testis is the androgen, testosterone, there are some reasons for believing that œstrogens may also be secreted in small amounts. Thus œstrone and œstradiol-17β have both been isolated from stallion testes, while the latter œstrogen has also been detected in the human testes. It is possible also that the testis may elaborate progesterone, since pregn-5-en-3β-ol-20-one has been isolated from swine testis tissue and since it has been shown that the testis possesses an enzyme system capable of oxidizing this steroid to progesterone. Various 'physiological inactive' steroids closely related in structure to testosterone and progesterone have also been isolated from testis tissue. Testosterone, like progesterone, gives rise to a considerable number of different metabolic products after it is secreted, which include at least three 3-ol-17-ones, two 3,17-diones and one 3,17-diol.

*The adrenal cortex.* — The state of affairs in regard to the adrenal cortex is even more complex. Seven different $C_{21}$ α-ketolic $\Delta^4$-3-ketones which are active in respect of

(*) This term is here used to describe those steroids which have not so far been shown to possess any physiological activity. It is possible that certain 'physiologically inactive' steroids may eventually be shown to possess physiological activities which are unsuspected at the present time.

electrolyte and/or carbohydrate metabolism have been isolated from the adrenal glands of animals, and there is evidence that at least three of these are secreted by the human adrenal. In addition to these hormones there is good evidence that the human adrenal cortex secretes œstrogens, progesterone and various $C_{19}$ androgens, while the presence of numerous 'physiologically inactive' allopregnane and $\Delta^4$-pregnene derivatives in the adrenal glands of animals suggests the possibility that such 'inactive' steroids may also be secreted by the human adrenal.

The metabolism in the tissues of the $C_{21}$ α-ketolic adrenocortical hormones after they are secreted gives rise to a considerable number of different types of steroid which are excreted in the urine. The $\Delta^4$-3-ketone group undergoes partial or complete reduction with the formation of one or two new asymmetric centres; the C-20 ketone group can be reduced to a secondary alcoholic group with the formation of a further asymmetric centre, and this can occur both with and without concomitant reduction of the primary alcoholic group of the side chain to a methyl group. Furthermore, it would appear that the 11 keto and 11 β-hydroxy steroids in this series are to some extent metabolically interconvertible; while those with a tertiary hydroxyl group at C-17 can to some extent undergo oxidative removal of the side-chain with the production of $C_{19}$ 17-ketosteroids, among which it may be noted are the three 3-hydroxy-17-ketones which are urinary metabolic products of testosterone. It will be apparent, therefore, that a single adrenocortical hormone, as for instance hydrocortisone, can give rise to a considerable number of different metabolic products of different types.

From the foregoing it will be clear that whether one wishes to use blood or urine steroid determinations to assess the secretory activity of one particular gland, or whether one wishes to use them to study the production or metabolic fate of one particular steroid hormone, the desirability of attempting to determine several different steroids or groups of steroids should at least be given some consideration. It would, of course, be ideal if every steroid originating in the particular gland or arising by the metabolism of the particular steroid hormone could be determined. However, this ideal state of affairs is not attainable with present techniques, and even if it were it would not be practicable for reasons of convenience.

In practice a compromise between attempting to do too much and too little has to be made, and the point at which one compromises must be determined by the availability of satisfactory quantitative methods for the different individual steroids or groups of steroids which might be determined, by the relative quantitative importance of these different individual steroids or groups of steroids, and, of course, by the kind of information which is being sought.

*The relative merits of blood and urine determinations*

Following the administration of certain steroid hormones to human subjects only small and somewhat variable proportions of the doses administered can be recovered from the urine in the form of identifiable metabolites or in the form of the unchanged hormones (*). Such findings suggest that the amounts of the steroid hormones and their metabolites which are excreted in the urine, may represent in these cases only small and variable fractions of the amounts of those hormones which are secreted in the body. Because of this some authorities have questioned the value of urinary steroid hormones as a means of assessing the secretory activities of the steroid hormone-secreting glands and have urged that blood determinations are to be preferred where suitable methods are available.

While the drawbacks of a urinary steroid determination as a means of accurately assessing hormone secretion must be admitted, there would seem to be no justification for regarding a blood determination as a preferable alternative for this purpose. A urine determination will yield a value which may be accepted as bearing some approximate proportionality to the total amount of hormone secreted during a certain period of time — usually from 8 to 24 hours. On the other hand, a blood determination will provide information about the amount of hormone or its metabolites which are present in the blood at one particular instant of time — the instant when the blood sample is withdrawn. This information may, of course, be just as valuable as, or in some circumstances even more valuable than that provided by a urine determination, but it must be emphasized that it is information of a different kind. Accordingly blood and urine determinations should not be regarded as alternatives, whose relative merits can be argued about, but rather as sources of different kinds of information which may be supplementary to one another.

In any case it would hardly be profitable at the present time to discuss the relative merits of the two types of determination, since there are as yet so few blood steroid methods which have any claims to be considered as quantitative procedures. The method for blood 17-hydroxycorticosteroids devised by Nelson and Samuels has undoubtedly proved its usefulness, and, considered as an analytical procedure, is probably as good as any of the methods for urinary corticosteroids. Promising preliminary work has been carried out by Zimmermann, Gardner, Samuels and his coworkers, and others on the determination of blood 17-ketosteroids, and doubtless a satisfactory method will shortly be available. On the other hand there is as yet no satisfactory method for determining blood œstrogens, and, notwithstanding certain claims made a few years ago, there is no satisfactory method for any of the progesterone metabolites in blood. However, a number of laboratories are undoubtedly alive to the importance of developing satisfactory methods for determining these and other steroids in blood, and accordingly the future can be looked forward to with some optimism.

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(*) This is the case with œstradiol-17β, œstrone and progesterone. It may apply, however, to a lesser extent for œstriol, testosterone and some of the adrenocortical hormones.

*A consideration of some general principles of methodology*

Among those who are actively concerned with the development and use of methods for the determination of blood and urine steroids there are two rather sharply divided schools of thought. On the one hand there are those who do not worry unduly about the specificity, accuracy and precision of the methods which they employ. Provided a method is rapid and simple to operate, and provided it gives high values in cases where high values are to be expected and low values when low values are to be expected, they are content. Those belonging to this school of thought are usually not steroid biochemists primarily but clinical chemists with hospital responsibilities. It is argued by them that such a method, even though it be relatively non-specific, inaccurate and unprecise, gives the clinician all the information that he requires. On the other hand there are the perfectionists to whom high specificity, accuracy and precision are all-important, and rapidity and simplicity of operation of secondary importance only. They are, for the most part, academic biochemists primarily interested in problems of steroid metabolism to whom a non-specific, inaccurate and unprecise method might be worse than useless. Many of them argue that even in the clinic, specific, accurate and precise methods should be used, even though that would involve the expenditure of considerably more time; that results obtained by the cruder methods may give the clinician misleading information; that it is a waste of time to devise such methods; and that it is bordering on scientific dishonesty to use them when their deficiencies are apparent.

While admitting the weight of the arguments advanced by those belonging to the first school of thought. I must say emphatically that my own sympathies are to a large extent with the perfectionists. It must be remembered that, with certain exceptions, the value of blood and urine steroid determinations for clinical purposes is still a subject for controversy amongst clinicians. It may be that many of these determinations would be useless for diagnostic or prognostic purposes even using analytical methods of proved specificity, accuracy and precision. However, until such methods have been devised and tested out in the clinic the value of many of these determinations must necessarily remain in doubt. In this connection I would like to quote a sentence from the introductory remarks by Dr. Gregory Pincus to Volume IX of *Recent Progress in Hormone Research*. He writes: « I recall many attempts to devise quick and easy methods for urinary estrogen or 17-ketosteroid determinations; and I ask myself if the time and energy spent in taking what have so often eventuated as fruitless short-cuts might not have been better spent in applying more laborious but fundamentally sound methods ». With equal justification Dr. Pincus might have said the same about urinary pregnanediol and corticosteroid determinations.

*Specificity.* — Most of the procedures commonly used for the actual measurement of steroids in blood or urine extracts are non-specific ones. For example the Zimmermann reaction is not specific for 17-ketosteroids; the production of a yellow colour with sulphuric acid is not specific for pregnanediol; and the formaldehydogenic and reduction reactions are not specific for the α-ketolic corticosteroids. Accordingly the specificities of methods in which such non-specific reactions are employed for individual steroids or for clearly defined groups of steroids must depend upon the procedures used for the purification and fractionation of the crude extracts.

It would not be profitable to compare the relative merits of methods specific for individual steroids with those specific for clearly defined groups of steroids, since obviously the choice between these two types of methods must depend upon the kind of information that is sought. For example, a determination of the total urinary 17-ketosteroids by one of the modifications of the Zimmermann-Callow procedure may often provide all the information that is needed; while in other circumstances it may be more informative to determine individual 17-ketosteroids by using the Zimmermann reaction subsequent to a chromatographic separation on a column or on paper. It shoud be emphasized, however, that whichever type of method is employed, whether in the research laboratory or in the clinic, a high degree of specificity is desirable : and I would add that in my personal opinion any compromise on this point is dangerous and is to be deplored.

*Accuracy and precision.* — It goes without saying that the accuracy of any analytical method ought to be tested before the method is used by determining the percentage recovery of pure substances previously added to the material to be analysed. In the present connection this would involve carrying out recovery experiments on blood or urine after the addition of pure steroids in the forms in which they are normally present. This, however, is difficult to do, and in most cases virtually impossible, because of the presence in blood and urine of conjugated forms of the steroids. In few instances is there any exact knowledge of the nature of these conjugates, and even in these few instances the pure conjugates are difficult to obtain. Accordingly in testing the accuracy of a steroid method the best one can do in most cases is to carry out recovery experiments after the addition of pure unconjugated steroids to the blood or urine (*). This procedure can provide one with a fair idea of the extent of the inevitable losses which occur during extraction and fractionation of the extracts, and it is a justifiable compromise, provided it is always borne in mind that it is a compromise.

It is impossible to lay down any hard and fast rules about the permissible limits of accuracy in a steroid method : and all I will say about this matter is that most of us who are interested in steroid methodology feel very satisfied if we can devise a method in which the recovery of added pure steroids is 75 % or better. I must, however, express my emphatic personal opinion that the employment of a method by a worker who has not himself determined its limits of accuracy by recovery experiments is seldom justifiable.

(*) This, of course, does not apply to those few methods in which the steroids are measured in conjugated form.

It is, of course, also essential for a worker to determine with his own hands the precision of any method he proposes to use, either by carrying out multiple determinations on the same sample of blood or urine, or by multiple recovery experiments with the same concentration of added steroid. Here again it is impossible to lay down any hard and fast rules, but in my opinion a standard deviation of ±10 % at optimal steroid concentrations is the best that one can reasonably hope for with most of the methods in use at the present time; and with some methods, particularly at sub-optimal steroid concentrations, one may have to be content with an S.D. of ±25-30 %.

*Hydrolysis of the urinary conjugated steroids*

In most of the methods commonly employed for the determination of urinary steroids it is necessary to hydrolyse the conjugated steroids (glucuronides and sulphates) before the urine is extracted. The classical method of doing this is, of course, by heating the urine with strong mineral acid. Hydrolysis in this way has been employed for many years for the determination of urinary œstrogens, pregnanediol and 17-ketosteroids. However it has its drawbacks. There is evidence that the hot acid treatment causes some loss of œstrogens and pregnanediol by decomposition to unknown products, although these losses are perhaps not so great as some have supposed; while in the case of the 17-ketosteroids various artifacts of known composition are formed.

Hot acid treatment cannot be employed in the determination of the α-ketolic corticosteroids, since the quantitatively most important of these, such as tetrahydrocortisone, are highly labile to this treatment because of the tertiary hydroxyl group at C-17 which they possess. However, a few years ago this difficulty was more or less satisfactorily overcome when it was found that steroids of this type could be liberated from their conjugated forms by incubation of urine with β-glucuronidase preparations.

While β-glucuronidase hydrolysis is undoubtedly a good method for the liberation of the 17-hydroxycorticosteroids from their conjugates in urine it is doubtful if it should yet be regarded as an entirely trustworthy method for use in the quantitative determination of these steroids : it is possible that variable amounts of β-glucuronidase inhibitors and of competing substrates may be present in urine, and it is also possible that a larger proportion of the 17-hydroxycorticosteroids may be conjugated as sulphates than is believed to be the case at present.

Accordingly, while the use of β-glucuronidase hydrolysis in the determination of the urinary corticosteroids may be justified on the grounds that it is by far the best method of hydrolysis available, it should be borne in mind that it may prove to be less quantitative than many at present believe it to be.

Some workers have considered the substitution of β-glucuronidase hydrolysis for hot acid hydrolysis in the determination of urinary œstrogens and pregnanediol, hoping by this means to avoid destruction of the steroids by the hot acid and to obtain extracts less contaminated by gums and pigments. In view of the possible untrustworthiness of β-glucuronidase hydrolysis the wisdom of doing this at the present time is doubtful.

It has been known for many years that some molluscs are a rich source of sulphatases, and recently workers in a number of laboratories, impressed by the fact that these molluscan sulphatases will hydrolyse dehydroepiandrosterone sulphate, have explored the possibility of using them to hydrolyse urinary steroid sulphates as a preliminary to the quantitative determination of the latter. However, considerable caution in the use of these sulphatases should be exercised, since the stereochemical specifities of these enzymes are peculiar. Thus last year Savard *et al.* showed that the sulphatase of *Otala punctata* would hydrolyse the sulphate of dehydroepiandrosterone but not that of androsterone; while more recently my colleague Dr. A. B. Roy has been able to make the important generalization, based on experiments with a large number of steroid sulphates, that the sulphatase of *Patella vulgata* (the common limpet) will hydrolyse the 3-sulphates of the 3 β-hydroxy-5 α steroids and those of the 3 β-hydroxy-$\Delta^5$ steroids, but will not hydrolyse those of the 3 β-hydroxy-5 β steroids nor those of the 3 α-hydroxy steroids whether of the 5 α or 5 β series. It is abundantly clear, therefore, that although the sulphatases of *Otala* and *Patella* may be valuable tools for the determination of certain urinary steroid sulphates they cannot be used for the hydrolysis of all urinary steroid sulphates.

Another difficulty involved in the use of these sulphatases for the hydrolysis of the urinary steroid sulphates, but not an insuperable one, is that these enzymes are markedly inhibited in the presence of phosphate or sulphate ions.

*The quantitative determination of œstrogens and pregnanediol in urine*

To conclude this introductory talk I would like to refer briefly to two quantitative steroid methods which have been recently developed by a certain of my associates in Edinburgh. This seems to me to be a good way of summarizing our general philosophy concerning steroid determinations.

Some years ago a group of us, representing various clinical and scientific departments in the Medical School, decided that we would embark upon a really thorough investigation of the normal menstrual cycle with a view to a subsequent study of menstrual abnormalities. At the outset we decided that an important part of this investigation should be a quantitative study of the urinary excretion of œstrogens and of progesterone metabolites throughout the cycle by chemical methods of high specificity and of satisfactory accuracy, and, of course, of sufficient sensitivity. At that time, however, such methods were not available, and accordingly the first task was to devise them.

*Oestrogens.* — For the œstrogens it was hoped that a method capable of determining œstradiol-17 β, œstrone and œstriol might be developed, since the possibility had to be considered that the relative proportions of the three might provide information of interest and value.

After several years of painstaking work, Dr. J. B. Brown, working in our Medical Research Council Unit,

was able to develop a method which seemed to meet our requirements. In this method, in which there are several novel procedures for the separation and purification of the œstrogen fractions, the final measurements are made by means of a modified Kober reaction (*), using a colour correction to eliminate errors due to non-specific brown colour.

Dr. Brown's method is normally carried out on 200 ml. samples of menstrual cycle urines. Experiments carried out with pure œstrogens added to the urine of men indicate that at concentrations of 5 μg. or more of each of the three œstrogens the recoveries average about 85 % with a standard deviation from the mean of about 6 %. The evidence put forward by Dr. Brown himself that the substances measured by his method in menstrual cycle urines are in fact œstriol, œstrone and œstradiol-17 β was not entirely conclusive. However, the specificity of the method as applied to such urines has recently been carefully examined by Diczfalusy in Stockholm using counter-current methods, and it has been shown beyond any reasonable doubt that it is these three œstrogens which are being measured.

It is undeniably a somewhat exacting and time-consuming method, but nevertheless it can be used as a routine day to day procedure in a well-organized laboratory. In fact it has been so used in Dr. Brown's own laboratory for over two years, and three trained technicians working eight hours per day for five days per week are regularly able to complete thirty determinations per week.

*Pregnanediol.* — As far as metabolites of progesterone are concerned we did not consider it necessary to attempt to determine anything other than pregnanediol, since the latter is by far the most important quantitatively of these metabolites. Up to a few years ago the method we used for pregnanediol was developed from the procedures described in 1944 by Astwood and Jones and by Talbot *et al.* This method appeared to be satisfactorily accurate and precise at pregnanediol concentrations above about 2.5 mg. per 24 hour urine specimen, but at lower concentrations its accuracy and precision were unsatisfactory, while furthermore we had some doubts about its specificity. It was felt, therefore, that a better method should be developed for our menstrual cycle investigations.

Dr. A. Klopper, also of our Medical Research Council Unit, has recently been successful in developing a method which seems in all respects to be far superior to any that have been previously described. It involves several new procedures, including chromatography on alumina after acetylation, for the purification of the pregnanediol in the urine extracts, and the final measurement is carried out by a modified version of the usual sulphuric acid colour reaction. By infrared spectra and by melting point and mixed melting point determinations it has been shown that the finally purified product upon which the colour reaction is performed contains pregnane-3 α, 20 α-diol diacetate and nothing else. It can be claimed, therefore, that the method is absolutely specific for pregnane-3 α, 20 α-diol.

Experiments with pure pregnanediol added to urine showed that at pregnanediol concentrations as low as 0.5 mg. per 24 hour urine sample the average recovery was 92 % with a standard deviation from the mean of about ±13 %. At higher pregnanediol concentrations the average recovery was found to be little higher, but the precision was improved.

This method is also exacting and time-consuming, but in Dr. Klopper's laboratory three technicians are normally able to complete some seventy determinations per week.

(*) Although much less sensitive than the fluorescence reaction, the Kober reaction has the great advantages of being more specific and of being less affected by impurities in the oestrogen containing fractions.

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## Some critical remarks about the methods for the determination of steroid hormones in blood and urine

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(*Received* 1 *August* 1955)

I quite agree with what Prof. Marrian has said with regard to the relative merits of blood and urine determinations. I believe, too, that we must not put the question in such a way as though determinations in blood would simply be preferable to determinations in urine. To illustrate this the following may serve :

In a few cases we determined the Porter-Silber chromogens both in plasma (with the method of Nelson and Samuels) and in urine (with the method of Reddy, Jenkins and Thorn) on the same day (table I).

It is evident that the discrepancy between the quantities of Porter-Silber chromogens in plasma and urine is often quite remarkable, especially in patients suffering from Cushing's syndrome. In patients suffering from Addison's disease on the other hand, we found low amounts of Porter-Silber chromogens, both in plasma and in urine. The explanation of this phenomenon may not exclusively be found in the facts already mentioned by Prof. Marrian. With the method of Nelson and Samuels only the 'free' Porter-Silber chromogens are determined ; therefore we must take account the possibility that the discrepancy arises from the fact that in plasma the

TABLE I

*Analysis of urine and plasma for Porter-Silber chromogens, by the method of Reddy, Jenkins and Thorn, and by the method of Nelson and Samuels*

| Sample | Plasma (μg./100 ml.) | Urine (mg./24 h.) | Clinical diagnosis |
|---|---|---|---|
| De. ♀ | 31.5 | 11.0 | Cushing's syndrome |
| Zo. ♀ | 14.2 | 15.1 | Cushing's syndrome |
| Ko. ♀ | 13.6 | 11.6 | Cushing's syndrome |
| Va. ♂ | 0.0 | 0.0 | Addison's disease |
| Pr. ♂ | 1.8 | 0.0 | Addison's disease |
| Vi. ♂ | 0.0 | 0.0 | Addison's disease |
| Bl. ♀ | 5.8 | 7.7 | Adrenogenital syndrome |
| Gh. ♀ | 14.5 | 2.4 | Hirsutism |
| Ba. ♂ | 8.7 | 2.6 | Epilepsia |
| Br. ♀ | 13.1 | 4.8 | Asthenia |
| Kl. ♀ | 14.0 | 2.4 | *Osteogenesis imperfecta* |
| Ja. ♀ | 44.0 | 11.9 | Tumour of the hypothalamus |
| Ap. ♀ | 37.2 | 1.2 | Tumour of the hypophysis |
| Bu. 1. ♀ | 41.1 | 4.5 | Primary amyloïdosis |
| Bu. 2. ♀ | 21.9 | 2.7 | Primary amyloïdosis |

The normal values are :
plasma : 3-16 μg. hydrocortisone/100 ml.
urine (female) : 1.2-8.6 mg. cortisone/24 h.
urine (male) : 2.9-12.0 mg. cortisone/24 h.

'conjugated' Porter-Silber chromogens, if present, were not determined. As things are we prefer the determination in urine as a support for the clinical diagnosis and as a check during a hormone therapy. We apply the determination in plasma when testing the adrenal cortical function with the help of ACTH. But even in this case the determination in urine can be made use of without causing serious difficulties. A combination of blood and urine determinations however, sometimes renders undeniably very interesting results. Thus with a patient suffering from primary amyloidosis we repeatedly found high values in the plasma, whereas low or normal amounts were found in the urine.

I should like to make another remark with regard to the hydrolysis of the conjugated steroids in urine. Several authors have already pointed out that the hydrolysis of the conjugated 17-ketosteroids by boiling the urine with strong mineral acid during 10 or 15 minutes, does not render satisfactory results. Lieberman recommends extraction of the acidified urine at room-temperature after which the urine residue is treated with β-glucuronidase. Many however, consider this undoubtedly superior method unfit for clinical purposes. In our laboratory we have applied hydrolysis of acidified urine at 80° C. for many years. During the hydrolysis, which is continued for 4 or 5 hours, benzene is used as extraction-agent. This procedure is based on a method published in 1938 by Dingemanse, Borchardt and Laqueur. A few years ago we compared this method with a method by which the hydrolysis is performed by heating at 100° C. during 10 minutes (1). We often found considerable differences.

It has also been established that the extracts prepared by heating the urine at 80° C. contain a higher amount of β-17-ketosteroids. With our routine method we found in the urine of normal men amounts of β-17-ketosteroids ranging from 0.8 to 11.6 mg. (the average being 4.5) and in the urine of normal women amounts from 0.2-5.9 mg./24 hours (the average being 1.5). These values are much higher than those we find in the literature. The ages of these normal subjects varied from 15 to 50 years. Since the original methodology as described by Dingemanse is time consuming and so rather unsuitable for routine purposes, we have recently tried to modify the method. For routine determinations we now add 5 ml. HCl 25 % and 25 ml. benzene to 50 ml. urine in a Kjeldahl flask of 500 ml. which is fitted with a coldfinger condenser and heated in a steambath during 5 hours. After cooling and separation of the layers we apply a second extraction with benzene. There are however indications that this second extraction can be omitted. I believe that Dr. Devis from Louvain, too, is in possession of data which point in the same direction. It is undoubtedly possible in this way to develop an extraction method which is not too complicated to be used in a clinical laboratory and yet gives results quite close to those of the more elaborate methods with respect to the quantitative extraction.

A few words about a question raised by the isolation of the i-androstanolone from urine by Dingemanse. According to several American investigators this substance is an artefact produced by hydrolysing dehydroepiandrosterone-sulphate in neutral medium. Meanwhile we succeeded in isolating a steroid-sulphate from the urine of several patients with tumours of the adrenal cortex; after hydrolysis of this substance in neutral medium it was possible to isolate i-androstanolone. The sulphate had a melting-point of 163°-165° C. and showed no depression of the melting-point when it was mixed with synthetic dehydroepiandrosterone-sulphate which had a melting-point of 160°-164° C. (*). The infra-red spectra of the two compounds were identical. On account of these results, we must come to the conclusion that the substance isolated from the urine is dehydroepiandrosterone-sulphate and that i-androstanolone is indeed an artefact formed during the hydrolysis in a neutral medium.

I want to make a few remarks about the extraction of Porter-Silber chromogens from urine. It is possible completely to avoid the hydrolysis-problem when extracting these substances by applying the method described by Reddy, Jenkins and Thorn. We have obtained much experience with this method after Dr. Bekaert from Ghent had drawn our attention to the fact that excellent results can be obtained with this methodology in the clinical laboratory. Table II gives an impression of the results of duplicate analyses.

(*) Dr. Klyne from London supplied us with the latter product.

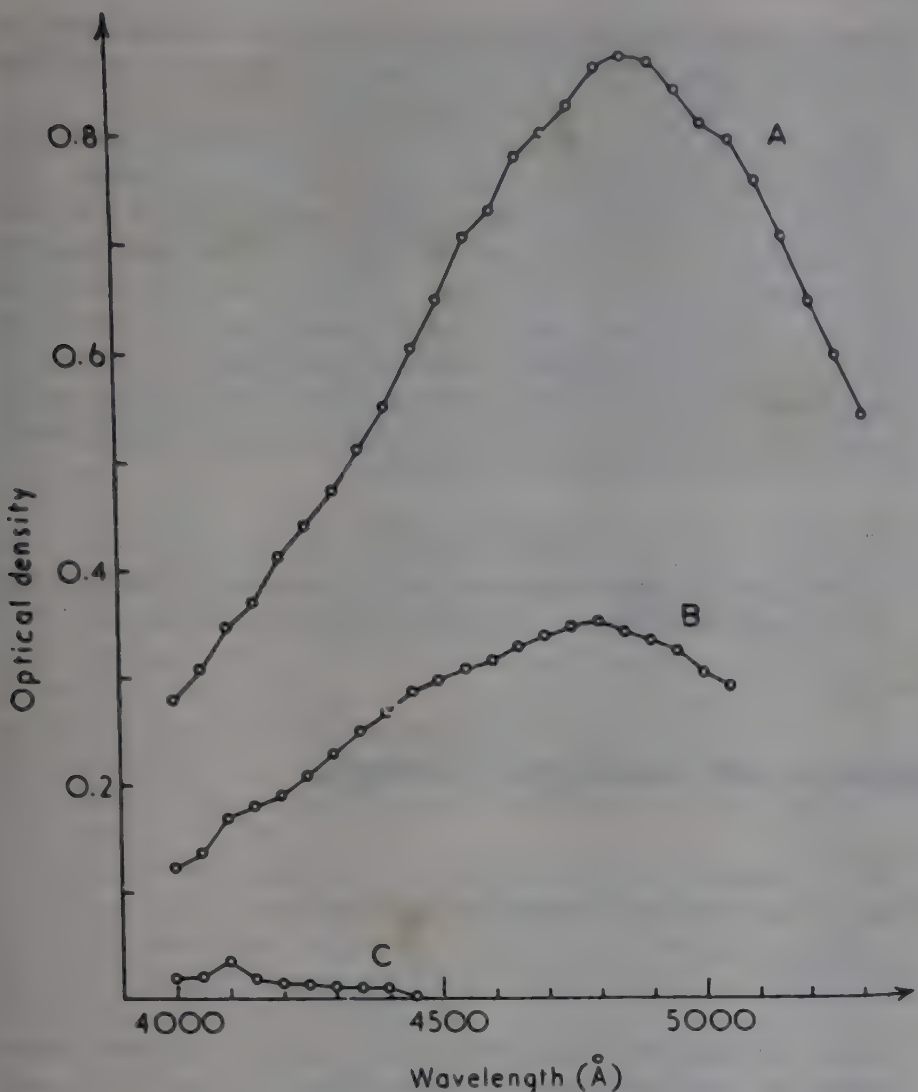


Fig. 1. — Absorption spectra of coloured solutions produced by phenylhydrazine-sulfuric acid with 1 ml. butanol extract (1 ml. urine) of : (A) a patient treated with 4 g. sulfamerazine/24 hours during some time ; (B) a normal male who took 4 g. sulfamerazine on the day preceding the recollection of the urine ; (C) the same normal male in a period during which no sulfamerazine was administered. All the readings are corrected for the colour produced by the entracts with sulfuric acid alone. Coleman Jr. 6A spectrophotometer.

Unfortunately it is impossible to test the accuracy of this method in an adequate way, because we have no pure conjugated corticoids at our disposal. We tried to form an opinion about the specificity of the Porter-Silber-reaction when it is applied to butanol extracts of urine. We are under the impression that human urine normally only contains Porter-Silber-chromogens derived from the adrenal cortex ; for the butanol extracts of urine from patients suffering from Addison's disease and from those suffering from destruction of the hypophysis contain in many cases no substances rendering a specific reaction.

In the literature however a list of therapeutic agents has already been published which have an effect on the result of the determination of Reddy *et al.* To this list we were able to add an excretion product of sulphamerazine and of sulphadiazine, which was until now not identified. The urine of patients treated with sulphamerazine or sulphadiazine contains a compound which gives an orange coloured solution with the phenylhydrazine sulphuric acid reagent. This solution gives a broad absorption band with a maximum at 4850 Å.

Table II

*Duplicate analyses of urine for Porter-Silber chromogens by the method of Reddy, Jenkins and Thorn*

| Sample | Porter-Silber chromogens (mg. cortisone/24 h.) | | Difference (in mg.) |
|---|---|---|---|
| D. M. | 11.4 | 11.2 | 0.2 |
| J. M. | 57.0 | 55.0 | 2.0 |
| J. T. | 2.8 | 1.3 | 1.5 |
| J. Me. | 15.1 | 15.1 | 0.0 |
| J. Te. | 0.0 | 0.0 | 0.0 |
| G. J. | 11.4 | 12.3 | 0.9 |
| d. W. | 0.0 | 0.0 | 0.0 |
| Me. | 7.2 | 7.6 | 0.4 |
| E. B. | 2.1 | 0.5 | 1.6 |
| Wij. | 9.0 | 10.4 | 1.4 |
| Hu. | 8.2 | 8.2 | 0.0 |
| v. D. | 0.7 | 0.5 | 0.2 |
| v. S. | 6.3 | 5.7 | 0.6 |
| Ne. | 7.8 | 8.1 | 0.3 |
| v. B. | 0.0 | 0.0 | 0.0 |
| Dij. | 3.8 | 4.8 | 1.0 |
| De. | 9.7 | 9.0 | 0.7 |
| v. O. | 7.1 | 8.2 | 1.1 |
| Ku. | 3.8 | 4.4 | 0.6 |
| Nij. | 7.7 | 6.6 | 1.1 |
| Nijp. | 4.1 | 3.7 | 0.4 |
| H. B. | 3.8 | 3.6 | 0.2 |

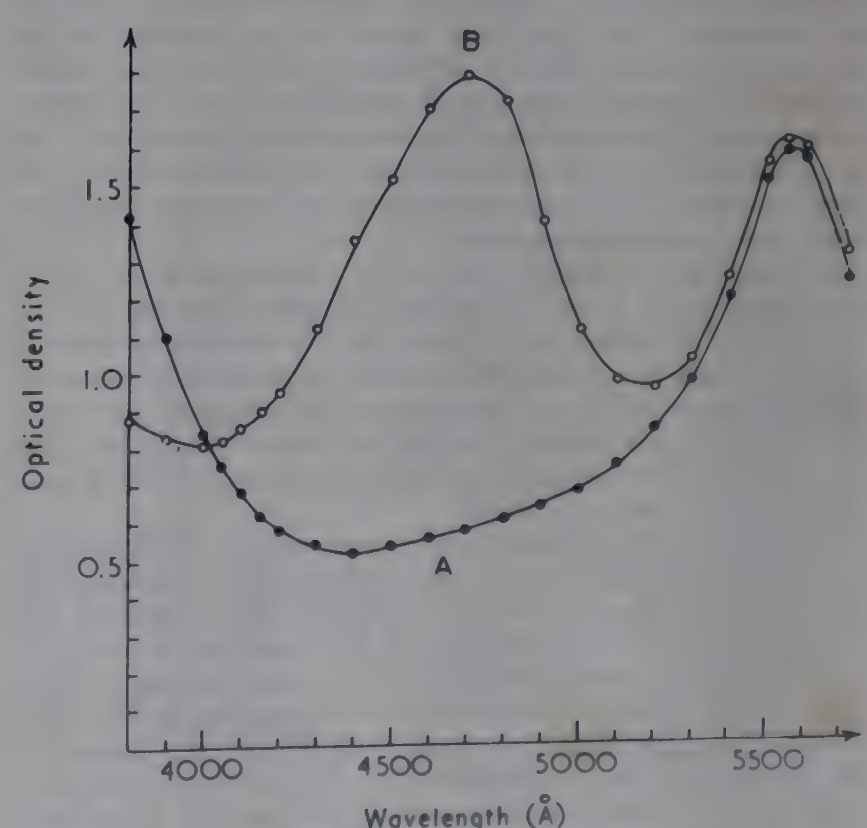


Fig. 2. — Absorption spectra of coloured solutions produced by the butanol entract of a patient treated with 9 mg. cortisone and 6 g. impure glycyrrhetinic acid. (A), with phenyl hydrazine-sulfuric acid ; (B), with sulfuric acid alone. Beckman Quartz spectrophotometer.

Because of the width and the intensity of this band it is impossible to eliminate this atypical colour.

After treatment with licorice extract the urine contains a compound, which also interferes with the determination of the Porter-Silber chromogens. Patients treated with licorice extract excrete compounds which develop red and orange colours in 60 % $H_2SO_4$. At first we thought these substances might be the metabolites of glycyrrhetinic acid. But on further investigation this proved not to be the case. The substance which causes an absorption band at 4600 Å in 60 % sulphuric acid reacts with the phenylhydrazine sulphuric acid reagent while forming an almost colourless complex. For these reasons it is impossible to determine the amount of Porter-Silber chromogens in these urinary extracts.

The above-mentioned experiences induced us to require full information about therapeutics administered, when receiving samples of urine for the determination of the amount of Porter-Silber chromogens according to the method of Reddy, Jenkins and Thorn.

My last remark concerns the form in which the Porter-Silber chromogens occur in urine. Only a small part of these compounds consists, as is known, of free cortisol and cortisone. The rest of the Porter-Silber chromogens are present in conjugated form. Part of these conjugates can be decomposed with β-glucuronidase or enzymes occurring in the digestif fluid of molluscs. After the treatment with enzymes however there is still a quantity of conjugates left in the urine which are not decomposed by the above mentioned enzymes.

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## Définition de la spécificité analytique en biochimie clinique

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(*Reçu le 5 août* 1955)

Dans son excellent rapport sur le dosage des stéroïdes dans le sang et l'urine le Professeur Marrian ouvre lui-même la discussion en opposant la philosophie des chimistes purs qui appliquent strictement les lois de l'analyse chimique, à celle des médecins biologistes qui se contentent de résultats imprécis, approximatifs et inexacts. Je pense que le problème doit être posé différemment et que les règles de la méthodologie des stéroïdes ne sont pas rigoureusement les mêmes dans un laboratoire de recherche sur le métabolisme de ces substances et dans un laboratoire spécialisé dans l'exploration fonctionnelle des glandes endocrines.

Le premier principe que nous discuterons est celui de la spécificité d'une technique. Je suis d'accord avec le Professeur Marrian sur le caractère de spécificité absolue que doit requérir une méthode analytique dans la recherche scientifique ; je pense néanmoins qu'imposer une telle exigence à la biochimie clinique relèverait d'un rigorisme qui risquerait d'éliminer de l'analyse médicale la grande majorité de ces méthodes.

Prenons comme exemple le dosage des 17-cétostéroïdes. A ma connaissance tous les auteurs qui mesurent ces métabolites dans les urines utilisent des méthodes très comparables. Elles consistent à extraire par un solvant organique les urines hydrolysées et, après lavage approprié, à appliquer à l'extrait final la réaction de Zimmermann. La spécificité de ces techniques ne repose ni sur la réaction de Zimmermann ni sur le procédé d'extraction utilisé. Est-ce à dire que le dosage soit à rejeter ? Un tel ostracisme serait en opposition avec le crédit qui lui est unanimement accordé en endocrinologie. Si nous en analysons les raisons, nous constatons que cette crédibilité repose sur une étude statistique des résultats obtenus dans des groupes de sujets normaux, en fonction de leur âge et de leur sexe. On en retire dans chaque groupe la notion d'une constante biologique du taux de ces métabolites dont la déviation standard fixe les écarts physiologiques. En appliquant la même méthode statistique à des groupes de malades rigoureusement catalogués on met en évidence la valeur séméiologique des 17-cétostéroïdes urinaires dans les deux secteurs de leur déficit et de leur excédent. Les zones de l'excrétion physiologique et pathologique de ces métabolites sont ainsi délimitées. Une méthode dont la spécificité analytique peut être considérée comme très faible acquiert ainsi en biochimie clinique une valeur séméiologique indéniable, fondée sur une notion de spécificité relative qui est établie, à l'égard d'un liquide biologique donné, par des critères physiologiques et pathologiques. Il s'agit là d'une méthode de travail que nous croyons fondamentale pour juger de la valeur d'un procédé en biochimie clinique, qu'il s'agisse du dosage du glucose ou de l'urée dans le sang ou de la mesure d'un stéroïde urinaire. Nous en donnerons un autre exemple. Celui du dosage des glycuronides de 3 α-stéroïdes (3 α-OH) non oxygénés en 11 ; nous avons remarqué avec Mlle Crépy que ces métabolites étaient extractibles par le butanol en milieu fortement alcalin et que le procédé était très spécifique surtout si l'on procède à deux extractions successives. Cette fraction de glycuroconjugués peut être mesurée avec précision et exactitude. La récupération d'un glycuronide de 3 α-stéroïde ajouté aux urines est excellente. Après administration de testostérone ou de progestérone la totalité des métabolites est retrouvée dans cette fraction de glycuroconjugués, que nous dénommons GBS 13. En appliquant à cette technique la méthode de travail et de pensée que je viens d'exposer pour les 17-cétostéroïdes nous avons pu démontrer la constance des résultats chez les sujets normaux en fonction de l'âge, du sexe et du cycle ovarien. En la pratiquant dans

des cas d'endocrinopathies bien sélectionnés et dans des expériences métaboliques avec des hormones stéroïdes ou des stimulines nous avons établi sa valeur séméiologique. Nous en donnerons un exemple, celui du diagnostic de la sénescence placentaire au terme de la grossesse où le taux de ces métabolites est généralement compris entre 80 et 130 mg.; dans tous les cas où nous l'avons trouvé inférieur à 45 mg., dans les deux derniers mois de la grossesse, nous avons pu constater la dégénérescence du placenta dont le diagnostic biochimique est fondé sur la constatation de cette corrélation entre une grandeur biochimique mesurable et une anomalie anatomique du placenta.

En revanche, il n'est pas possible de tirer un verdict aussi précis de la détermination spécifique du pregnandiol dont l'amplitude des variations à cette époque de la grossesse est beaucoup plus grande que celle des 3 α-stéroïdes totaux. Là encore, c'est l'étude objective et statistique des résultats qui donne à une méthode, non spécifique du point de vue de la définition analytique, une valeur séméiologique plus grande qu'à une technique dont la spécificité repose sur un point de fusion ou un spectre infra-rouge.

Ce serait méconnaître les impératifs qu'imposent à la technique l'exploration endocrinienne que de supposer que toute méthode, si compliquée et si coûteuse soit-elle, peut être utilisée dans un laboratoire d'analyse médicale du seul fait de ses qualités de précision, d'exactitude et de spécificité. Il faut savoir qu'une méthode qui peut donner d'excellents résultats dans les mains d'un chimiste très qualifié peut, du seul fait de sa complexité, entraîner des erreurs graves lorsqu'elle est confiée à un technicien moins averti qui ne travaille pas dans la sérénité et la tranquillité d'un laboratoire de recherche.

Mais ce n'est pas tant sur cet écueil que nous désirons mettre l'accent que sur la polyvalence des données qu'exige l'exploration d'une glande endocrine. Pour explorer la corticosurrénale il faut, à notre avis, effectuer avant et après perfusion d'ACTH, les déterminations de la créatinine, des 17-cétostéroïdes, des 17-OH-stéroïdes et éventuellement de la folliculine. Pense-t-on qu'un tel travail pourrait être réalisable sous l'angle de la routine clinique si l'on ne disposait de méthodes d'exécution suffisamment rapides? Mon expérience personnelle me permet d'affirmer, sur un très grand nombre de résultats, que les renseignements donnés par les méthodes que nous avons standardisées à l'Ecole de Puériculture sont parfaitement valables et qu'ils apportent aux cliniciens une symptomatologie biochimique statique et potentielle de la corticosurrénale.

Est-ce à dire que l'analyse médicale doive renoncer à la précision et à l'exactitude ? J'estime que les méthodes dont la déviation standard, calculée sur un grand nombre de résultats obtenus en double, dépasse 10 à 15 % ne doivent pas être acceptées et qu'on ne peut faire de concession sur la précision et l'exactitude d'un procédé. Lorsqu'une méthode a répondu favorablement à ces critères analytiques et aux expériences de récupération d'un métabolite approprié ajouté aux urines, c'est à des critères physiologiques et pathologiques qu'il faut avoir recours pour apprécier sa spécificité et pour porter un jugement sur sa valeur en biochimie clinique.

Si on prend l'exemple du dosage de la folliculine, pour lequel les Docteurs Brown et Bauld ont mis au point des techniques très remarquables, je ferai les remarques suivantes : *a*) il ne paraît pas nécessaire en biochimie clinique de doser séparément les trois œstrogènes urinaires car ils varient dans le même sens physiologiquement comme pathologiquement; *b*) on apprécie parfaitement les variations physiologiques et pathologiques de ces métabolites en les évaluant globalement, avec une méthode sans doute moins spécifique mais dont l'exactitude et la précision répondent aux règles ci-dessus énoncées. C'est ce que nous avons constaté avec notre nouvelle méthode de dosage de la folliculine urinaire qui est adaptée à la routine clinique.

C'est l'hydrolyse diastasique des conjugués par le suc gastrique d'*Helix pomatia* qui constitue une de ses améliorations principales. En ajoutant, par ml. d'urine, 1000 unités glycuronidasiques et 1500 à 2000 unités sulfatasiques nous avons trouvé sur une cinquantaine d'échantillons urinaires que l'hydrolyse des œstrogènes conjugués était quasi complète en 18 heures lorsque le taux de la folliculine était inférieur à 1 mg./litre. Nous n'avons pas noté d'inhibition par des substances urinaires à ces hautes concentrations en diastases.

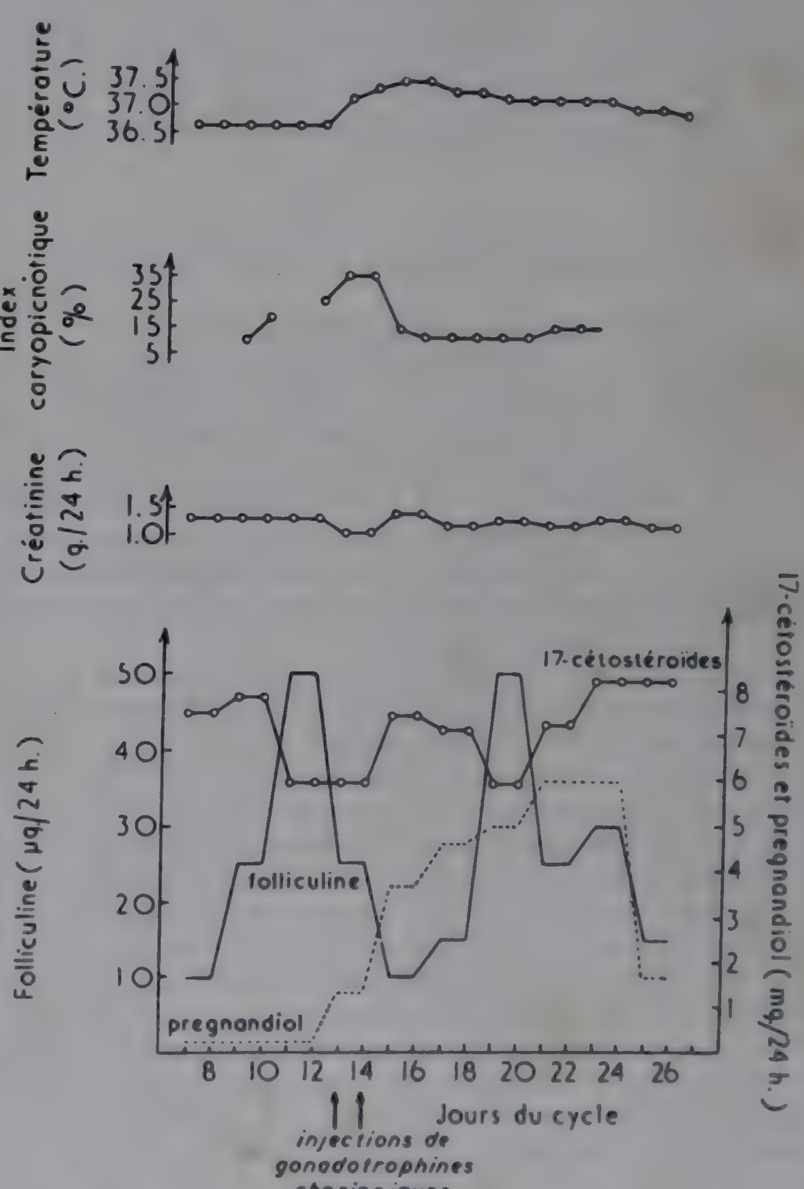


FIG. 1. — Élimination des stéroïdes au cours d'un cycle menstruel (administration de 10 000 unités de gonadotrophines chorioniques le 13e et le 14e jours).

TABLEAU I

*Réponses normales à l'administration de 30 000 unités internationales de gonadotrophines chorioniques, en 3 jours, au milieu du cycle menstruel*

| Sujet | Avant | du 1er au 2e jour | du 3e au 4e jour | du 5e au 6e jour | du 7e au 8e jour | |
|---|---|---|---|---|---|---|
| R. V., 37 ans | 10 | 25<br>0<br>D. T.(*) | 10<br>+4.2 | 15<br>+4.4 | 50<br>+5.6 | folliculine (μg./24 h.)<br>Δ prégnandiol (mg./24 h.) |
| M. P., 35 ans | 15 | | D. T.(*) | 25<br>+3 | 55<br>+5 | folliculine (μg./24 h.)<br>Δ prégnandiol (mg./24 h.) |
| Pr., 34 ans | 10 | 15<br>+4 | D. T.(*) | | 35<br>+6 | folliculine (μg./24 h.)<br>Δ prégnandiol (mg./24 h.) |
| Ba., 31 ans | 5 | | D. T.(*) | | 45<br>+10 | folliculine (μg./24 h.)<br>prégnandiol (mg./24 h.) |
| Po., 25 ans | 10 | 15<br>+1 | 10<br>+3 | | 140<br>+7<br>D. T.?(*) | folliculine (μg./24 h.)<br>prégnandiol (mg./24 h.) |
| Go., 22 ans | 10 | 5<br>0 | 0<br>0 | | 85<br>+2 | folliculine (μg./24 h.)<br>prégnandiol (mg./24 h.) |
| Des., 43 ans | 5 | | | | 50<br>+8.5<br>D. T.(*) | folliculine (μg./24 h.)<br>prégnandiol (mg./24 h.) |

(*) D.T. = décalage thermique au dessus de 37°C.

Nous avons fait la même constatation pour le prégnandiol dont le dosage spécifique, par la méthode de Mlle Crépy, peut-être couplé avec le précédent.

L'hydrolyse enzymatique possède une grande supériorité sur l'hydrolyse chlorhydrique ; elle évite la destruction des stéroïdes qui est particulièrement importante dans le cas de la folliculine ; elle diminue considérablement la quantité des pigments urinaires éthéro-solubles. Elle augmente la spécificité de la réaction de Kober appliquée à l'extrait phénolique final.

Sur la figure 1 on peut juger de l'influence du cycle ovarien sur la courbe de l'excrétion folliculinique chez un sujet normal. Lorsque 20 000 U. I. de gonadotrophines chorioniques sont administrées on voit le taux de la folliculine s'élever à plus de 50 μg. une semaine plus tard. Cette méthode est à la base d'un test dynamique de l'exploration de l'ovaire. Le tableau I rend compte de la permanence de l'élévation de la folliculine au huitième jour après la première injection, entre 40 et plus de 100 μg. En revanche, cette élévation ne se produit pas en cas de destruction organique ou d'absence de l'ovaire. La figure 2 montre l'incidence d'une grossesse au début sur l'excrétion des œstrogènes. Le tableau II montre l'influence de l'âge et du sexe sur le taux nycthéméral de la folliculine urinaire. On voit au cours du cycle menstruel que sur les urines prélevées le 4e et le 5e jour après le décalage thermique, soit en plein milieu de la phase lutéale, on trouve en moyenne 27 ± 9 μg., alors que du 7e au 10e jour du cycle le taux moyen est seulement de 16 ± 9 μg. Dans l'aménorrhée la moyenne

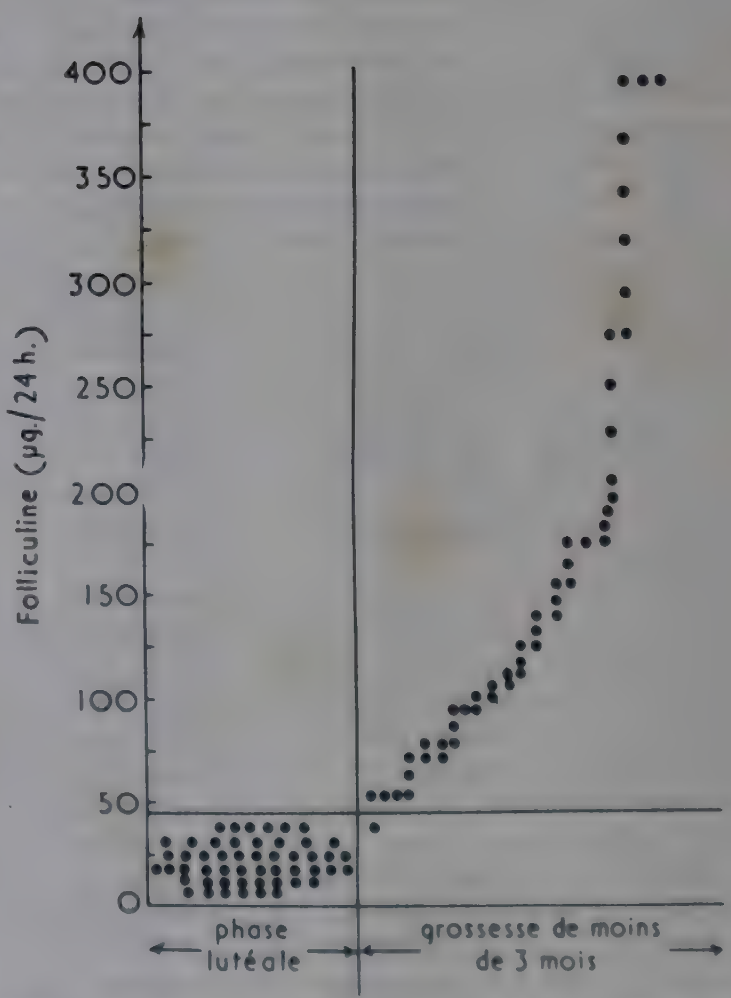


FIG. 2. — Élimination des œstroïdes au cours de la grossesse.

TABLEAU II

*Taux nycthéméral de la folliculine urinaire* (μg.)

| | Nombre de cas | Moyenne ± écart-type |
|---|---|---|
| Enfants : | | |
| 1-6 ans | 14 | 3 ± 1.2 |
| 6-12 ans | 42 | 7 ± 3.4 |
| Hommes : | | |
| 20-45 ans | 10 | 13 |
| Femmes : | | |
| phase folliculaire | 84 | 16 ± 9 |
| phase lutéale | 15 | 27.2 ± 9.01 |
| Aménorrhées | 47 | 8.6 ± 6 |

tombe à 8 ± 6 μg. Ainsi, collectivement comme individuellement, on peut conclure, en utilisant ces critères physiologiques, que notre méthode présente une spécificité suffisante pour détecter les quantités de folliculine produites par l'ovaire aux trois périodes de son évolution cyclique, c'est-à-dire à la période de repos, à l'époque de l'ovulation et au milieu de la phase lutéale. Comme nous l'apprend une étude statistique il est également possible de faire le diagnostic d'états hyperfolliculiniques en pathologie ovarienne et surrénale et ainsi d'apporter à l'endocrinologie une séméiologie biochimique qui est encore améliorée par l'administration de stimulines appropriées.

Le temps et la place nous manquent pour apporter des arguments symétriques sur l'intérêt physiopathologique du micro-dosage du prégnandiol réalisé après hydrolyse enzymatique par la microméthode de Mlle Crépy. Nos recherches cliniques nous apprennent également que cette méthode spécifique n'est pas suffisante pour l'exploration de la fonction lutéale et qu'il est utile de pratiquer également des déterminations des 3 α-OH pendant les deux phases du cycle pour avoir, par différence, une évaluation des métabolites totaux d'origine lutéale.

Je voudrais conclure en disant que la biochimie clinique implique l'adaptation de l'analyse chimique aux objectifs recherchés par le physiologiste et le clinicien. Il est nécessaire de la pratiquer avec le double point de vue d'un analyste et d'un pathologiste pour comprendre sa philosophie, ses exigences et ses particularités ; elle est une science expérimentale relativement autonome qui possède sa doctrine et sa méthodologie et qui repose sur des principes analytiques mais aussi, pour une grande part, sur des critères physiologiques et pathologiques que fournit l'étude statistique des résultats chez des groupes bien sélectionnés de sujets normaux et malades.

## Steroid analysis

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(*Received* 11 *August* 1955)

Professor Marrian is to be complimented on his excellent presentation of the problems confronting those of us who are interested in quantitative determinations of steroids in blood and urine. The speaker has properly emphasized the point that blood and urine methods should not be regarded as alternatives but rather supplementary to one another. It is conceivable that urine analysis of a particular metabolite could indicate a decreased daily production by a specific gland while the blood determination could indicate a normal value. This situation could arrive if the rate of disappearance of the steroid hormone from blood is decreased. On the other hand, it is possible to find an increased urinary excretion of steroid hormone metabolites and a normal level of blood hormone if the rate of disappearance of the hormone is increased. Such considerations are not merely theoretical possibilities in view of the recent findings of Peterson and Wyngaarden (1) who showed increased blood disappearance rates of cortisol in thyrotoxicosis patients and decreased rates in patients with liver diseases.

It is impossible to overemphasize the importance of the concept that steroid determinations in blood and urine are of value only if we understand the metabolism of the steroid hormone in question. It is essential that tissue hormones be carefully related to specific metabolites in the blood and in the urine. A good start has been made in this direction but a great deal remains to be done. The availability of labeled steroids has made the task a great deal simpler.

The importance of the knowledge of steroid metabolism for the interpretation of steroid analysis has been discussed by the speaker who gave a pertinent example in discussing the many metabolites of cortisol. It is perhaps to the point to discuss briefly the determination of androsterone and etiocholanolone in urine of men. These metabolites may derive from testosterone (I), (probably produced exclusively from the testis), from $\Delta_4$-androstene-3,17-dione (II) (principally from the adrenal), and from dehydroepiandrosterone (III) (perhaps exclusively from the adrenal). A measure of these two compounds in urine gives a value related to the formation and metabolism of all three steroids (I, II, III) rather than to any single tissue hormone. It is not surprising, then, that over the years castrated men have been found whose urinary titers of 17-ketosteroids and even andro-

gens (determined by biological means) were normal. Actually, no direct urinary method is available to assess the male hormone production by the testis. Further studies of the differential metabolism of I, II and III are needed to achieve this objective.

I would like to mention, briefly, two recent methods which have been developed at the Worcester Foundation which make it possible to obtain valuable information from studying urinary steroids.

First, Burstein, Dorfman, and Nadel (2) used ethyl acetate, instead of the less polar solvents such as ether, benzene, chloroform, carbon tetrachloride, and methylene chloride, which have been used previously, to extract the steroids from urine of guinea pigs and of a human subject. For the first time, a group of steroids more polar than 3 α, 11 β, 17 α, 21-tetrahydroxypregnan-20-one were isolated. One of these steroids was identified as 6 β-hydroxycortisol. This is the first demonstration of *in vivo* 6 β-hydroxylation in humans, and its significance in pathological and stress conditions can now be studied.

Second, Dr. Marrian briefly mentioned that methods are available that are specific for the determination of the individual steroids. Rubin, Dorfman, and Pincus (3) have published a method for determining seven of the individual 17-ketosteroids of urine, utilizing a paper chromatographic separation. It is possible to measure quantitatively 3 β-chloro-$\Delta^5$-androsten-17-one, androsterone, etiocholan-3 α-ol-17-one, androstane-3 α, 11 β-diol-17-one, etiocholane-3 α, 11 β-diol-17-one, androstan-3 α-ol-11,17-dione, and etiocholan-3 α-ol-11,17-dione, when there is as little as 2.5 mg. total 17-ketosteroid. Mean total recovery of α-17-ketosteroids calculated from a series of 59 analyses was 98 ± 1.3 %.

The method is practicable for the study of urines of subjects receiving steroid therapy, where compounds not normally found in urines are present. For example, 6 mg. per day of testosterone were found in the urine of a woman receiving 300 mg. per day of testosterone. The testosterone appeared in the paper chromatograms as a discrete zone which in no way interfered with the usual steroid zones.

The method is applicable, also, when abnormal concentrations of some of the hormones cause extreme skewing of the normal pattern. Such a condition may be found in pathological states, such as diseases of the adrenal cortex, or may be artificially produced by the administration of steroid hormones. Accumulation of a mass of reproducible information about the urinary excretion products arising on administration of the various endogenously produced steroids of the adrenal cortex and the testis has enabled Dorfman (4) to formulate generalizations about the relationships between adrenal steroids and their urinary metabolites. Using these generalizations, it has been possible, at least in a preliminary way, to assess the production of the steroids by the gland, from the concentrations of the end products in the urine. This type of analysis has been applied to a study of the function of the adrenal gland in the separate clinical states : Cushing's syndrome, the adrenogenital syndrome, and adrenal carcinoma (masculinizing type) and to suggest working hypotheses concerning possible specific metabolic defects in the adrenal in these different types of hyperactivity.

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# Biosynthetic mechanisms of the formation of organic acids in moulds

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(*Received* 27 *June* 1955)

### *General Considerations*

Most of the published research on carbon metabolism in moulds has been concerned with the formation of simple acids, many of which, such as citrate, are also intermediates in the carbon metabolism of bacteria, higher plants, and animals.

Numerous theories have been advanced for the mechanism of their biosynthesis, but unequivocal experimental proof for these theories has only been obtained in a few cases. The most significant advances have been made only in recent years through the application of the radioisotope techniques. The present review is chiefly concerned with a review of this work. The pioneering work of Raistrick and his colleagues (for review, see 1) has revealed the existence of a large number of mould metabolites formed from glucose, many of acid nature, which are characteristic for moulds only.

Raistrick and his colleagues have, in many cases, succeeded in establishing the chemical structure of these substances, but very little is as yet known about the mechanism of their biosynthesis. Application of the isotope techniques should provide valuable information on this problem, which represents a hitherto almost completely unexplored and rewarding field of research.

The investigator in the field of mould metabolism is confronted by several difficulties which do not exist to the same extent in the field of bacterial metabolism.

The two greatest of these difficulties are :

— The lower fungi are morphologically more complex than the bacteria and their growth can occur in different morphological forms, depending on the experimental conditions. The different forms may differ significantly in their metabolism. This fact, of particular importance in submerged cultures, has not been sufficiently taken into account in most publications.

— Very little work on isolated enzyme systems in cell free extracts of lower fungi has been reported, because of the difficulty of obtaining active extracts. This difficulty is partly due to the presence in the cell extracts of powerful proteolytic enzymes which have their maximum activity at or near pH 7. These enzymes are very active even at 2° C., and dialysis at this temperature and pH 7 may lead to loss of 80 % of the protein nitrogen. The proteolytic enzymes present in extracts of *Penicillium chrysogenum* may be inhibited by a mucoprotein fraction of egg white (2).

Metabolic studies on lower fungi are carried out in surface or submerged culture. Attention has already been drawn by Kluyver and Perquin (3) to the non-homogeneity of the conditions of surface growth, with the concomitant poor reproducibility of the metabolic results. In their classical paper they proposed the method of submerged growth in shake flasks as an alternative for obtaining more homogeneous conditions of growth and better reproducibility of the metabolic reactions ; this 'shake flask' method is now the most widely used for the study of mould metabolism. It is not realized by all workers in the field of mould metabolism that in surface cultures after a few days growth, only a thin layer on the surface of the mycelial felt contains hyphae in a fully vital condition ; the rest of the mycelium (up to 90 %) consists of dead cells in various stages of autolysis.

There can be no doubt that conditions of submerged growth are much better suited for metabolic studies than those of surface growth. However, many filamentous fungi show different morphological forms of growth in submerged culture, depending on the culture conditions. Thus, submerged growth of *P. chrysogenum* can occur in pellets, in long filaments, and in a short branched form (4, 5). The short branched form contains per unit dry weight many more apical ends than the long filamentous form ; this manifests itself in differences in metabolic behaviour, as the growing apices have a different metabolism from the resting older cells. Other filamentous fungi show a similar variety of morphological forms in submerged culture. Steel, Martin and Lentz (6) report that *Aspergillus niger* can grow in the filamentous and pellet form ; the morphological appearance of the latter varies with age and culture medium. These authors consider that one particular form of pellets is the most suitable one for citric acid production (see also 7).

Among the factors which interfere with the type of growth in submerged culture one of the most important is the intensity of the mechanical agitation which is used in stirred fermenters for air dispersion. There

exists, within a certain range, an almost linear relationship between oxygen diffusion rates and the power consumption for mechanical agitation (8), (see the same paper for the measurement of oxygen diffusion rates by means of the rotating platinum electrode). The effect of the shearing forces brought about by mechanical agitation on the mycelium is of great importance and has not been sufficiently taken into account in previous work. Mechanical agitation induces branching in *P. chrysogenum* and prevents the formation of pellets.

Considerable differences in penicillin titres were noticed when propellers for air dispersion were used which had different sizes and rotated at different speeds, but gave the same oxygen diffusion rates; higher yields were obtained with a large propeller rotating at a lower speed than with a smaller propeller rotating at a higher speed, the oxygen diffusion rates being identical in each case (5). An effect of mechanical agitation on kojic acid production by *Aspergillus flavus* was also noted (9). Increase of the agitation intensity led to a decrease of the kojic acid production and an increase in a starch like material in the mycelium. Characteristic morphological changes in the appearance of the mycelium were noted to be induced by mechanical agitation, manifesting themselves in the formation of short, highly branched, swollen hyphae. When metabolic studies with filamentous fungi are scaled up from shake flasks to small stirred fermenters consideration has to be given to the fact that the intensity of the mechanical agitation for air dispersion which is required to obtain adequate oxygen diffusion rates may induce profound changes in the metabolic reactions under investigation, and in some cases may lead to complete disappearance of the desired metabolite. The shearing forces per unit volume of culture fluid required for obtaining a given aeration rate decrease with increasing fermenter volume; it is sometimes possible to reproduce in large stirred fermenters shake flask conditions with regard to the morphological appearance of the hyphae, which cannot be obtained in smaller fermenters.

*Citric acid formation in* Aspergillus *and the citric acid cycle*

The production of citric acid by *A. niger* is an industrial process of considerable importance. This fact has for many years past stimulated a great deal of research into the biochemical mechanism of its formation, and now that it is realized that citric acid plays an important part in the mechanism of the oxidative breakdown of carbohydrates in animal tissues, the interest of biochemists in this problem has increased.

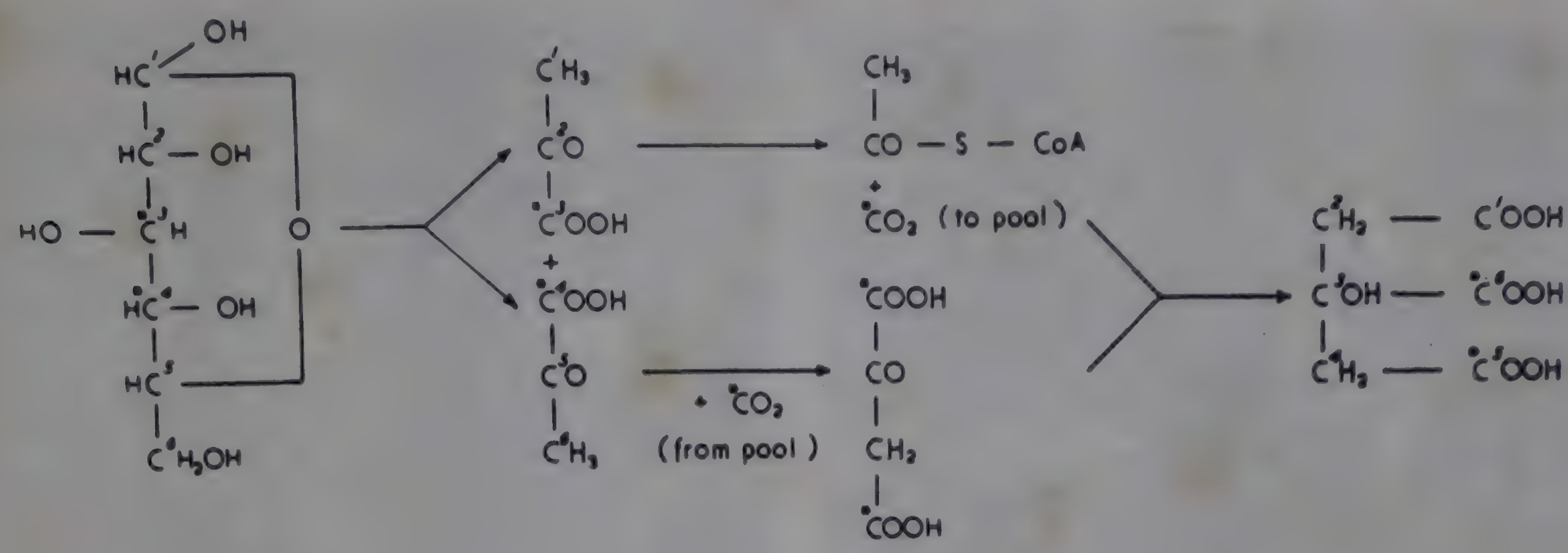
In 1919 Raistrick and Clark (10) suggested that the main mechanism for the formation of citric acid was the condensation of acetate and oxaloacetate. Much convincing, if indirect, evidence in favour of this theory was obtained by studies in which it could be shown that the yield of citrate was increased by the addition of acetate, pyruvate, and other substances acting as a precursor for the two or the four-carbon fragments (for literature up to 1949, see the excellent review of Walker, 11). Direct evidence has since become accessible by the use of isotopes, and there is no doubt that the mechanism proposed by Raistrick and Clark is correct.

Experiments with labelled precursors have been carried out by several investigators, using a variety of labelled substrates as follows : (*i*) $^{14}CO_2$ (12), surface growth, replacement cultures with acetate and mixtures of acetate and glucose as carbon source; (*ii*) $^{14}CO_2$ (13, 14), submerged growth in shake flasks, label added in replacement medium, sucrose as carbon source; (*iii*) $^{14}CO_2$ (15), aerobic submerged conditions, type of growth not described, replacement medium, sucrose as carbon source; (*iv*) $^{14}CO_2$ (16), pellets, rotary shaker, replacement medium, glucose as carbon source; (*v*) $H^{14}COOH$ (15), conditions as above; (*vi*) $^{14}C$ carboxyl and methyl labelled acetate as sole carbon source, and in conjunction with glucose (17), surface growth, label added in replacement medium; (*vii*) $^{14}C$ methyl labelled acetate (18), submerged aerobic conditions, type of growth not stated, acetate sole source of carbon, added in replacement medium; (*viii*) $^{14}C$ carboxyl labelled acetate (19), pellet growth in shake flasks, a trace of labelled acetate was added to glucose as sole carbon source; (*ix*) $^{14}C_1$- labelled glucose (20), pellet growth in shake flasks, glucose as sole carbon source; and (*x*) $^{14}C_3$, $^{14}C_4$- labelled glucose (16), pellet growth in shake flasks; glucose as sole carbon substrate.

As can be seen, the conditions of growth in the different experiments varied considerably, so that no direct comparison is possible. Only in the experiments of Bomstein and Johnson (19), Cleland and Johnson (16), and Shu, Funk and Neish (20), did a large accumulation of citric acid occur (appr. 50 to over 70 % of the glucose utilized). In all the other work the accumulation of citrate was of a low order.

The most convincing proof that acetate and oxaloacetate condensation is the main mechanism of citrate accumulation by *A. niger* has been provided by the elegant experiments of Cleland and Johnson. Using 3, 4 labelled glucose these authors found that the carbon of the tertiary carboxyl in citric acid contained the label of one of the carbons from glucose practically undiluted, some radioactivity was also found in 5-C, the carbon of one of the primary carboxyls. These clearcut findings are compatible only with a split of the glucose into two three-carbon fragments (presumably pyruvate), decarboxylation of one of them with the formation of a two-carbon fragment, carboxylation of the second three-carbon fragment to oxaloacetate, and condensation of the two-carbon fragment and the four-carbon oxaloacetate to citrate (figure 1).

Carbon 4 of glucose thus becomes carbon 6 of the citrate, with its specific activity practically undiminished; carbon 5 of the citrate shows some radioactivity, because carboxylation of one of the two pyruvates to oxaloacetate has taken place with $^{14}CO_2$ from the pool. The radioactive $CO_2$ deriving from the other of the two three-carbon compounds was diluted about seven times by the non-radioactive $CO_2$ from the respiring mycelium; the dilution of the label of 3-C in glucose, as found in 5-C of the citrate formed, is about sixfold. That carboxylation of the three-carbon compounds is the main mechanism of $C_4$ compound formation under the conditions of the experiment was demonstrated by the authors in the same paper by showing that on incubation of the cultures with $^{14}CO_2$ the citrate contained 88 % of its radioactivity in carbon 5; the remaining 12 % was



(•) = $^{14}C$ - labelled

FIG. 1. — Conversion of 3,4-$^{14}C$-labelled glucose into citrate (after Cleland and Johnson, 16).

| Specific activity of carbon atoms in glucose (c.p.m.) | |
|---|---|
| $C_1$ | 270 |
| $C_2$ | 270 |
| $C_3$ | 39 600 |
| $C_4$ | 39 600 |
| $C_5$ | 270 |
| $C_6$ | 27 |

| Specific activity of carbon atoms in citric acid (c.p.m). | |
|---|---|
| $C_1$ | 270 |
| $C_2$ | 270 |
| $C_3$ | 270 |
| $C_4$ | 270 |
| $C_5$ | 5 870 |
| $C_6$ | 39 050 |

located in the tertiary carboxyl, possibly due to equilibration with fumarate.

The experiments of Cleland and Johnson demonstrate conclusively that the citrate formed could not have been recycled through the tricarboxylic acid cycle, for if this had been the case the radioactivity of the tertiary citrate carboxyl would have been lost in the form of radioactive $CO_2$.

Furthermore, a '$C_3 + C_2$' condensation to form the four-carbon compound, which has been assumed to occur by various authors (for older literature, see 11 ; for a discussion of the formation of fumarate from ethanol by this pathway see below), is excluded, for any oxaloacetate formed by condensation of two two-carbon fragments from carboxyl labelled pyruvate would be without radioactivity.

In an earlier paper (19), in which a trace of carboxyl labelled acetate was added to glucose as sole carbon source, the authors came to a similar conclusion as to the mechanism of citrate accumulation. Under these conditions they found 70 % of the total radioactivity of the citrate in carbon 1, the rest about equally divided between carbons 5 and 6 ; carbons 2, 3 and 4 were inactive.

The high proportion of radioactivity in 1-C proves that '$C_2 + C_4$' condensation is the main mechanism of citrate formation under these conditions ; repeated cycling of the citrate is excluded, because the radioactivity in 5-C and 6-C is too low to have been derived by this mechanism.

Shu *et al.* (20) have studied the distribution of radioactivity in citrate formed from glucose-1-$^{14}C$ under submerged culture conditions similar to those of Cleland and Johnson ; their yield of citrate was high (over 60 %). The distribution of the radioactivity obtained is given in table I.

TABLE I

*Distribution of radioactivity in citrate formed from glucose-1-$^{14}C$*

| Citrate carbon | µC./g. carbon | % of mol. activity of $^{14}C$ in glucose-1-$^{14}C$ (18.84 µC./g. carbon) |
|---|---|---|
| All carbons | 3.70 | 19.6 |
| 1.5 | 1.13 | 5.99 |
| 6 | 1.37 | 7.25 |
| 2.4 | 6.71 | 35.6 |
| 3 | 3.99 | 21.2 |

After Shu, Funk and Neish (20).

As could be expected, the distribution of radioactivity is similar to that obtained by Lewis and Weinhouse (see below, table II) using $^{14}CH_3$ labelled acetate, *i.e.* the specific activity of carbon 2 and 4 was highest, and the tertiary carboxyl 6-C had a higher specific activity than the two primary carboxyls. The authors subjected these figures to a mathematical analysis and conclude that under their conditions 40 % of the citrate is formed from recycled $C_4$ acids, and 40 % from $C_4$ acids synthesized by '$C_3 + C_1$' condensations.

Their conclusions as to the main pathway of citrate biosynthesis thus differ from those which Cleland and Johnson drew on the basis of their clear-cut experimental results with 3,4-$^{14}C_2$ labelled glucose as tracer. It is, however, possible to interpret the results of Shu *et al.* in a different manner, and to arrive at conclusions which can be better reconcilied with those of Cleland and Johnson. It seems to the reviewer that, on the basis

of the quantitative data available, an unequivocal mathematical differentiation between the different possible pathways of citrate synthesis as proposed by Shu *et al.* is impossible without making a number of assumptions which cannot be checked experimentally.

It is obvious that the tricarboxylic acid cycle cannot operate completely if a large accumulation of citrate takes place. The question arises whether all its reactions occur under conditions in which citrate does not accumulate in large quantities. Such conditions prevailed, in fact, in the majority of the experiments on the mechanism of citrate formation by *A. niger* recorded in the literature in which isotopes were employed.

All these experiments strongly suggest that the '$C_2 + C_4$' condensation step postulated by Raistrick and Clark (10), and proved by Cleland and Johnson (16) to occur under conditions in which large amounts of citrate accumulate, also occurs under conditions in which citrate does not accumulate. Foster, Carson, Ruben and Kamen (21), using submerged cultures of *A. niger* in shake flasks, were the first to demonstrate that $^{14}CO_2$ is incorporated into the citric acid molecule and that nearly all the activity is present in the carboxyl groups. The distribution of radioactivity in the individual carboxyl groups was not investigated. In similar experiments Martin, Wilson and Burris (13) and Martin and Wilson (14) showed that $^{14}CO_2$ was incorporated to about an equal degree into the primary and tertiary carboxyl group of citrate, whereas the central carbon atoms 2, 3 and 4 were devoid of activity; Lewis and Weinhouse (12), using surface replacement cultures, obtained the same results, but were able to show by degrading the citrate to oxaloacetate and succinate that over 80 % of the radioactivity of the two primary carboxyl groups was present in one of them, 5-C.

All these results prove the formation of a four-carbon compound by carboxylation of a three-carbon compound, and the subsequent condensation of this four-carbon compound with a two-carbon compound to give citrate. The equal distribution of radioactivity between 5-C and 6-C is in contrast with the results of Cleland and Johnson (16) and suggests that under conditions in which citrate does not accumulate in large amounts equilibration between oxaloacetate and fumarate has taken place, with randomization of the radioactivity between the two carboxyl groups.

Evidence that some such mechanism is responsible for the appearance of radioactivity in 6-C is contained in the results of Mosbach, Phares and Carson (15), who have shown that, after 15 h. incubation of *A. niger* cultures with $^{14}CO_2$, the specific activity of 5-C is less than half that of 6-C, but that after 40 h. it increases to nearly 80 % of that of 6-C.

That cycling of citrate may occur under conditions in which its accumulation is low may also be deduced from the experiments of Lewis and Weinhouse (17) and of Carson, Mosbach and Phares (18) on the incorporation of label from $^{14}CH_3COOH$. The first authors worked with the surface culture and replacement medium, the second with submerged growth. Both groups of authors found incorporation of radioactivity in all carbon atoms of the citrate molecule, as shown in the following tables II and III.

**Table II**

*Incorporation of radioactivity from $^{14}CH_3COOH$ in citrate*

| Carbon number of citrate | Form in which isolated | $\frac{\text{Relative specific activity of substances (*)} \times 100}{\text{Activity of citrate (*)}}$ | |
|---|---|---|---|
| Overall | Ca citrate | 100 | 100 |
| 1 and 5 | $CO_2$ | 51 | 45 |
| 6 | $CO_2$ | 63 | 61 |
| 2, 3, 4 | $CH_3$-CO-$CH_3$ | 146 | 149 |
| 2 and 4 | $CHI_3$ | 165 | 135 |
| 2, 3, 4, 5 | Na acetate | 152 | 148 |
| 3 | $BaCO_3$ | 147 | 149 |

(*) Measured as $BaCO_3$.
After Lewis and Weinhouse (17).

**Table III**

*Incorporation of radioactivity from $^{14}CH_3$-COOH in citrate*

| Carbon number of citrate | Specific activity |
|---|---|
| 1 | 132 |
| 2 | 560 |
| 3 | 598 |
| 4 | 592 |
| 5 | 463 |
| 6 | 476 |

After Carson, Mosbach and Phares (18).

From the more equal distribution of the radioactivity in the citrate carbons in the experiments of Carson, Mosbach and Phares, it can be concluded that under their conditions rather extensive cycling of the citrate has taken place. The presence of activity in carbon 1 makes necessary the assumption that in some way

doubly labelled acetate must have been formed during the metabolism of $^{14}CH_3COOH$, possibly through formate formation (see below).

Lewis and Weinhouse (17) carried out incorporation experiments with carboxyl labelled acetate in addition to those with methyl labelled acetate; surface growth and a replacement medium were used. Radioactivity was found exclusively in the carboxyl groups, the ratio of activity of primary carboxyls/tertiary carboxyl varying between the values of 1 and 1.8 (no distinction between 1-C and 5-C was made in the experiments). These results, apart from giving supporting evidence for the occurrence of a '$C_2 + C_4$' condensation reaction in the citric acid biosynthesis, show that some process of cycling and randomization of the radioactivity between the carboxyl groups must have occurred. More evidence for cycling was obtained by Mosbach, Phares and Carson (15) using $H^{14}COOH$ as carbon source. About 80 % of the total radioactivity in the citrate molecule was recovered in 5-C and 6-C, about 8 % in 1-C, and the rest in 2-C, 3-C and 4-C, the latter showing the highest activity (about 8.7 %).

The present state of knowledge on citric acid biosynthesis by *A. niger* gained on the basis of isotope studies may, then, be summarized as follows : citrate accumulates through the condensation of active acetate and oxaloacetate; the oxaloacetate is predominantly formed by carboxylation of a three-carbon compound and '$C_3 + C_3$' condensation does not play an important part in its biosynthesis. When citrate does not accumulate, but is metabolized, recycling of citrate along the reactions of the tricarboxylic acid cycle probably takes place.

The two main intermediates postulated in citric acid biosynthesis through condensation of active acetate and oxaloacetate, *i.e.* acetate and pyruvate, have now been isolated from culture filtrates of *A. niger* (22); various cycle acids have also been isolated from culture filtrates in which no large accumulation of citrate occurred (α-ketoglutarate by Walker, Hall and Hopton, 23; succinate, malate, and fumarate by Halliwell, 24). The accumulation of pyruvate in the culture medium was achieved by addition of arsenite, which had the effect of stimulating glucose utilization and citrate formation, but repressed carbon dioxide production (25). The stimulating effect of arsenite on citrate production under these conditions may be due to the fact that, by stabilizing pyruvate, it makes it more available for citrate synthesis. Condensing enzyme, catalysing the condensation of active acetate and oxaloacetate, has been isolated from the mycelium of *A. niger* grown in shake flasks (26, 27); citric acid synthesis occurred readily in cell free extracts in the system used by Ochoa, Stern and Schneider (28), *i.e.* in the presence of coenzyme A, oxaloacetate, acetylphosphate, transacetylase and magnesium ions. In a later paper Ramakrishnan and Martin (29) report that magnesium ions were not required for the condensation, and were actually inhibitory.

An indication that the tricarboxylic acid cycle is interrupted under conditions in which citrate accumulates was obtained by Ramakrishnan, Steel and Lentz (30), who demonstrated that the activities (expressed in units/mg. of protein) of two enzymes of the cycle, aconitase and isocitric dehydrogenase, disappear during citric acid fermentation at the stage when citrate accumulation begins; the activity of condensing enzyme increased greatly under these conditions. These authors observed that isocitric dehydrogenase was completely inhibited by citric acid in concentrations of $4 \times 10^{-4}$ M.

The mechanism of the formation of pyruvate from glucose in the biosynthesis of citrate by *A. niger* has not been investigated in detail. Evidence for the presence of most of the enzymes of the Meyerhof-Embden glycolytic cycle has been obtained by Jagannathan and Singh (31); these authors showed that ammoniacal extracts of the mycelium of *A. niger* grown on a glucose medium in submerged culture contained the following enzymes : hexokinase, phosphoisomerase, phosphofructokinase, aldolase, phosphoglyceraldehyde dehydrogenase, phosphoglyceromutase, enolase, phosphopyruvic kinase, carboxylase, and alcohol dehydrogenase. The activity of some of these enzymes was, however, very low, and the extracts did not bring about glycolysis. The aldolase was further purified and shown to be a metalloprotein (32) which was inhibited by the chelating agent, ethylenediamine tetracetic acid; the inhibition could be reversed by addition of zinc ions.

The formation of citrate by *A. niger* is inhibited by iodoacetate, fluoride, or phloridzin both in normal surface culture and in replacement cultures (33). Cyanide, on the other hand, was found to stimulate citric acid formation (33, 34) and inhibit its breakdown (34), possibly through formation of cyanhydrin compounds with the α-ketoacids of the tricarboxylic acid cycle.

Oxalic acid has been known for a long time to accompany citrate acid production by *A. niger*. Several hypotheses for its formation have been advanced (see 11), but none of them has been conclusively proved. Experiments with isotopically labelled compounds have given valuable new information, but have not as yet led to the final elucidation of this interesting old problem. Lewis and Weinhouse (17), using surface cultures, found that carboxyl labelled acetate gave rise to considerable formation of radioactive oxalate (about four times the amount of citrate formed), and its specific activity was about the same as that of the citrate formed. This showed that acetate was a precursor of oxalate, a conclusion already arrived at by other investigators on the basis of the fact that addition of inert acetate to the culture medium notably increases the yield of oxalic acid (*e.g.*, 35; for older literature see 11).

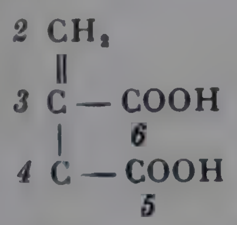
Experiments with $^{14}CO_2$ (12) showed that this compound was incorporated into oxalate, though to a lesser degree than into citrate. This suggests that oxaloacetate too may act as a precursor of oxalate. The activity of the oxalate was too low for the whole amount formed to have been derived from oxaloacetate through carboxylation; other pathways of oxalate formation must have been operative in addition.

Bomstein and Johnson, working with shake flask cultures of *A. niger*, showed that the oxalate formed from glucose to which a trace of carboxyl labelled acetate was added was radioactive; the activity, however, was only a small fraction (1.5 to 10 %) of that of the citrate formed simultaneously, and was too low to have been formed by direct oxidation of the acetate or from any

of the carboxyls of the citrate. Furthermore, the specific activity of the oxalate decreased during the fermentation, while that of the citrate strongly increased. While oxalate could have been formed from oxaloacetate or oxalosuccinate during the initial stages of the fermentation, it must have derived from other non-radioactive sources during later stages. Lewis and Weinhouse (12) added radioactive citrate (all the radioactivity resided in the carboxyl groups : 1229 c.p.m. in 1-C, 181 c.p.m. in 5-C and 685 c.p.m. in 6-C) to a surface culture actively metabolizing citrate, and isolated the oxalate formed under these conditions; it was radioactive, but much less (20 c.p.m.) than any of the carboxyl groups of the citrate. Though the endogenous metabolism of the mould was higher under the condition of the experiments of Lewis and Weinhouse, the authors concluded that the low activity of the oxalate excluded its formation by a direct split of oxalosuccinate, as postulated by Lynen and Lynen (36).

*Production of itaconic acid by* Aspergillus terreus

Itaconic acid :



is a metabolic product of *Aspergillus terreus* first discovered by Kinoshita (38). It is an unsaturated dicarboxylic acid, closely related to the tricarboxylic cycle intermediate aconitate, and has considerable theoretical interest. It may also have industrial importance as a starting material for polymers, and pilot plant processes on a semi-industrial scale have been worked out for its production (39, 40, 41, 42).

The mechanism of biosynthesis has been studied with the use of radioisotopes by Corzo (43).

He used $^{14}CH_3COOH$, $CH_3$-$^{14}COOH$, $^{14}CO_2$, and asymmetrically labelled citrate as tracer, with glucose as carbon source, in a synthetic medium in shake flasks. Growth occurred in pellet form. The yield of itaconic acid obtained was fairly high (about 25 % of the glucose metabolized). By means of a novel degradation method, Corzo was able to estimate the distribution of radioactivity in the individual carbon atoms of the itaconic acid molecule. Itaconic acid was cleaved with ozone in the presence of 2,4-dinitrophenylhydrazine, which gave 2-C as formaldehyde, 2,4-dinitrophenylhydrazone and the remaining four-carbon unit as the 2,4-dinitrophenylhydrazone of oxaloacetic acid. This was decarboxylated, giving 5-C in the form of $CO_2$, and 2-C, 3-C, and 6-C as the 2,4-dinitrophenylhydrazone of pyruvic acid. The latter, on oxidation with ceric sulphate, yielded 6-C in the form of $CO_2$ and the remaining two-carbon unit, 3-C and 4-C, as acetic acid; the combined activity of these two carbons was determined after combustion to barium carbonate.

The results of the tracer incorporation experiments are reported in tables IV, V, VI and VII.

TABLE IV

*Incorporation of asymmetrically labelled citric acid into itaconic acid*

| Carbon atom | % of total activity | |
|---|---|---|
| | Itaconic acid | Citric acid |
| All | 100 | .... |
| 5 | 48.9 | 52.4 |
| 3 and 4 | 14.8 | 3.2 |
| 2 | 0.6 | 0.0 |
| 6 | 41.3 | 41.3 |

After Corzo (43).

The distribution of label in the asymmetrically labelled citric acid is evident from the last column. The figure 3.2 in the third row refers to 3-C only; 1-C had 3.2 % of the total activity, 4-C no activity.

TABLE V

*Incorporation of $^{14}CH_3COOH$ in itaconic acid by* A. terreus

| Carbon atom | % of total radioactivity |
|---|---|
| All | 100 |
| 5 | 5.3 |
| 3 and 4 | 29.6 |
| 2 | 60.5 |
| 6 | 5.2 |

After Corzo (43).

TABLE VI

*Incorporation of $^{14}CO_2$ into itaconic acid by* A. terreus

| Carbon atom | % of total radioactivity |
|---|---|
| All | 100 |
| 5 | 53.8 |
| 3 and 4 | 16.1 |
| 2 | 2.1 |
| 6 | 35.4 |

After Corzo (43).

They indicate that most probably itaconic acid is derived from citrate by dehydration and loss of carboxyl 5. This is evident from the fact that the radioactivity of asymmetrically labelled citric acid (table IV), appears in the corresponding carbon atoms of itaconic acid in an almost identical quantitative distribution.

The pathway of biosynthesis in *A. terreus* of citric acid, the precursor of itaconic acid, is probably the same

**Table VII**

*Incorporation of $CH_3$-$^{14}COOH$ in itaconic acid by* A. terreus

| Carbon atom | % of total radioactivity |
|---|---|
| All | 100 |
| 5 | 45 |
| 3 and 4 | 15.6 |
| 2 | 9.5 |
| 6 | 28.4 |

After Corzo (43).

as that occurring in *A. niger* and discussed above, *i.e. via* a '$C_2 + C_4$' condensation. This is apparent from the results with methyl labelled acetate (table V) : most of the activity resides in 2-C, as expected. Extensive carboxylation is evidently taking place, as occurs in *A. niger*; this is demonstrated by the high radioactivity of 5-C and 6-C of the carboxyl groups, when $^{14}CO_2$ or $CH_3{}^{14}COOH$ is used as tracer (tables VI and VII). The presence of radioactivity in the central carbon chain in these experiments indicates that a certain amount of recycling has taken place, as it does in citric acid formation in *A. niger* when this acid does not accumulate in large amounts.

Eimhjellen and Larsen (44) found that in *A. niger* (see above), pyruvate accumulates in the presence of arsenite during the formation of itaconic acid by *A. terreus*. These authors obtained further evidence that citric acid or some activated form of citric acid is an intermediate in the biosynthesis of itaconic acid. They found, using shake flask cultures of *A. terreus* and replacement media, that itaconic acid was formed as readily from citrate as it was from glucose. When glucose plus citric acid were used as the carbon source, the glucose only was utilized for itaconic acid production, a finding which suggests that the active form of citric acid required for itaconic synthesis is more available from glucose than from added citric acid.

In contradistinction to citric acid, *cis*-aconitic acid was only very slowly or, in some experiments, not at all metabolized by the mould; and, in the cases where it was metabolized, the yield of itaconic acid did not exceed that produced by the endogenous metabolism.

This makes it unlikely that *cis*-aconitic acid is the immediate precursor of itaconic acid, as was originally postulated by the discoverer of this substance Kinoshita. Further evidence to this effect is the finding that the addition of flyroroacetate considerably stimulated the formation of itaconic acid from citric acid (from 18 to 63 %) and, to a lesser degree, from glucose. Fluoroacetate has been shown to exert its inhibitory effect in animal tissues by undergoing condensation to fluorocitrate, which has been demonstrated to be a specific inhibitor of aconitase (46, 47). If the mechanism of fluoroacetate inhibition of glucose metabolism in *A. terreus* is the same as that in animal tissues, *cis*-aconitic acid is excluded as a precursor of itaconic acid.

Larsen and Eimhjellen (45), studied the effect of pH on the production of itaconic acid from glucose. It had already been shown that production of itaconic acid takes place only under acid conditions (48). Larsen and Eimhjellen showed that mycelium grown under acid conditions was capable, in the resting condition in replacement media, of converting glucose into itaconic acid both at acid and neutral pH at about the same rates; mycelium grown at neutral pH, however, did not produce itaconic acid either in the growth-supporting original medium, or in replacement media at acid or neutral pH. Formation of itaconic acid could be induced by first growing the mould at pH 6 and than suspending it in a fully growth-supporting medium at pH 2.

It would appear that the enzyme system required for the transformation of glucose into itaconic acid is formed only under acid conditions of growth. When *A. terreus* is grown in neutral medium, more glucose is transformed in mycelium than when it grows at acid pH, and L-malate, succinate, and fumarate accumulate.

### *Oxidative metabolism of glucose and organic acid metabolism in* P. chrysogenum

The metabolic activities of *P. chrysogenum* are of special interest in connexion with its ability to produce the antibiotic penicillin. Several groups of workers have begun to study the mechanism of oxidative carbohydrate breakdown, but these investigations must still be considered to be in their beginnings only.

It has been known for some time that *P. chrysogenum* and *Penicillium notatum* possess an active glucose dehydrogenase, converting glucose into gluconic acid. The enzyme has been purified and shown to be a flavoprotein with alloxazine adenine dinucleotide as prosthetic group. Keilin and Hartree (49) showed that the enzyme has a very specific action on glucose, and can thus be used for the determination of glucose in biological materials. In a later paper the same authors showed that β-glucose is oxidized much more rapidly than the α-form (50); they also demonstrated the presence in the mycelium of *P. notatum* of a thermolabile, non-dialysable substance which actively catalyses mutarotation (51). Bentley and Neuberger (52), in studying the mechanism of glucose oxidation to gluconic acid by glucose dehydrogenase, obtained evidence that the first product formed was δ-gluconic lactone. The reaction is a typical dehydrogenation, the hydrogen from glucose being transferred to oxygen from the air to form hydrogenperoxide. This was conclusively proved by Bentley and Neuberger (52) by the use of $^{18}O_2$ and $H_2{}^{18}O$; in the former case the hydrogen peroxide formed, on decomposition with catalase, gave oxygen gas with the same $^{18}O_2$ abundance as the oxygen in the gas phase, while when the experiments were carried out on $H_2{}^{18}O$, but with $^{16}O_2$ in the gas phase, the oxygen formed from hydrogen peroxide on decomposition with catalase was found to be free of $^{18}O_2$.

Two main pathways of oxidative breakdown of glucose are known : one by way of the Meyerhof-Embden glycolytic cycle, leading to the formation of pyruvate, which is then oxidized by way of the tricarboxylic cycle; the other *via* oxidation of glucose-6-phosphate to phospho-

gluconate with subsequent decarboxylation, leading to the formation of a pentose phosphate, which in turn is split into a two- and a three- carbon compound, both of which are then oxidized. De Fiebre and Knight (53) have studied the oxidation of glucose-1-$^{14}$C and glucose-2-$^{14}$C by pellets of *P. chrysogenum* grown in submerged culture. They found that while with both types of labelled glucose most of the radioactivity was incorporated in the cells, about 30 % of the glucose-1-$^{14}$C appeared as carbon dioxide while only about 0.2 % of the total activity of the 2-$^{14}$C-labelled glucose appeared as carbon dioxide. This shows clearly that the labelled 1-C is readily eliminated as $CO_2$, indicating a decarboxylation of gluconic acid.

Furthermore, in alcoholic cell extracts both glucose-6-phosphate and 6-phosphogluconate could be identified. Koffler (54) has reported similar experiments and similar results ; he further demonstrated the presence of enzymes in cell free extracts which catalysed the oxidation of glucose-6-phosphate and phospho-6-gluconate in the presence of triphosphopyridinenucleotide. Evidence that in addition to the hexose monophosphate shunt there exists a glycolytic pathway of glucose oxidation was also obtained by Koffler, who showed that in the presence of arsenite glucose-1-$^{14}$C was partly converted in methyl labelled pyruvate with a specific activity in the methyl groups of about 1/3 that of the glucose.

### *Role of the tricarboxylic acid cycle in the oxidation of glucose*

The question whether the oxidation products of glucose, formed by the mycelium of *P. chrysogenum* by the hexose monophosphate shunt and Meyerhof-Embden glycolytic pathways, are oxidized *via* the tricarboxylic acid cycle, has been given attention by several investigators. Hockenhull, Herbert, Walker, Wilkin and Winder (55), found that several cycle acids, such as succinate, fumarate, and citrate, were oxidized at rates appreciably higher than the endogenous respiration by mycelium grown on a synthetic medium similar to that of Jarvis and Johnson (56), containing as carbon sources lactose, glucose, acetate and lactate.

TABLE VIII

*Oxidation of various cycle acids by starved mycelium (kept for 16 h. in phosphate) of* P. chrysogenum *grown on synthetic medium*

| Substrate | $Q_{O_2}$ total (uncorrected) | $Q_{O_2}$ corrected for endogenous respiration |
|---|---|---|
| Acetate | 6.10 | 3.40 |
| Lactate | 3.8 | 1.10 |
| Succinate | 5.55 , 5.50 | 2.85 , 2.80 |
| Fumarate | 4.65 , 4.70 | 1.90 , 2.00 |
| Malate | 6.20 , 6.70 | 3.50 , 3.40 |
| Citrate | 3.70 , 3.40 | 0.95 , 0.65 |
| None | 2.75 , 2.70 | 0 0 |

After Hockenhull, Herbert, Walker, Wilkin and Winder (55).

However, Olson and Chain (57), using mycelium grown on acetate as the sole carbon source, found that while acetate was very rapidly oxidized (with a $Q_{O_2}$ of 80 and above) fumarate, succinate, malate and citrate did not appreciably increase the endogenous respiration, which under the conditions of their experiments was very low (figure 2). With pyruvate and oxaloacetate a slight

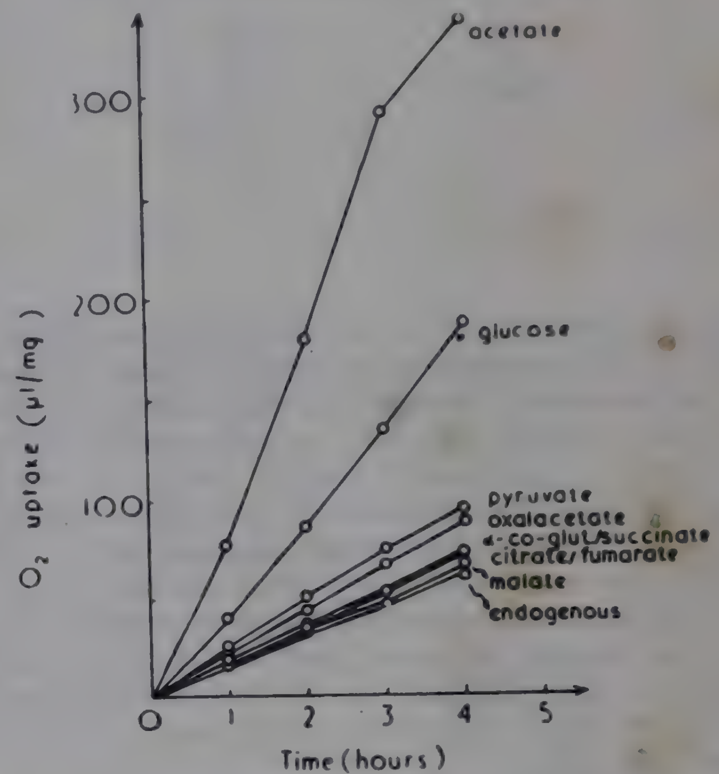



FIG. 2. — Respiratory activity of washed suspensions of mycelium. Each manometer flask contained 0.025 M substrate and 2 mg. (dry wt.) mycelial suspension in 3 ml. M Na phosphate buffer pH 6.8. Substrate was added 10 min. before the first readings. 0.2 ml. KOH (30 g./100 ml.) was placed in the centerwell and the suspension was incubated in air at 25° C. The bend in the acetate curve after 3 hours is due to exhaustion of substrate.

increase of the respiration rate above that of the endogenous respiration was obtained, but the increase was still very small by comparison with that produced by acetate. Similarly Beevers, Koffler and Goldschmidt (58) found that mycelium of acetate grown mould did not oxidize succinate, but they ascribed this to a permeability barrier, since the broken up mycelium was capable of oxidizing succinate. They suggested, as a means of overcoming the permeability barrier to dicarboxylic acids, the use of the esters instead of the free acids. In this way they found that the rate of endogenous respiration was about doubled after addition of 0.005 M diethyl succinate at pH 6. Furthermore, while malonate had no inhibitory effect on the oxidation of acetate, diethylmalonate did exert an inhibiting action. Olson and Chain (57) have confirmed that diethyl succinate is oxidized at a rate slightly above that of the endogenous respiration at pH 6 and pH 4, but much below that of acetate oxidation. They also confirmed the inhibitory effect of diethyl malonate on acetate oxidation, but found that this inhibition was not specific.

Acetate oxidation and even endogenous respiration was completely inhibited by the ethyl esters of fumarate, malate and citrate.

TABLE IX

*Effect of different esters on oxidation of acetate*

| Substrate | Percentage of maximal rate of acetate oxidation | |
|---|---|---|
| | pH 4 | pH 6 |
| Sodium acetate | 14 | **100** |
| Ethyl acetate | 5 | 10 |
| Diethyl succinate | 21 | 13 |
| Diethyl fumarate | Inhib. (*) | Inhib. (*) |
| Diethyl malate | Inhib. (*) | 0 |
| Triethyl citrate | Inhib. (*) | Inhib. (*) |
| Diethyl malonate | Inhib. (*) | Inhib. (*) |

(*) = Inhibition of endogenous respiration.

Experimental conditions : Warburg vessels containing the following final concentrations : 0.01 M acetate, 0.05 M ester substrate, 0.1 M universal buffer, 0.2 ml. 30 % KOH in center well. Flasks incubated at 25° C. for 60 minutes.

These experiments throw doubt on the usefulness of esters as a tool to overcome permeability barriers. A number of cases have been reported in which micro-organisms, which in the intact state did not attack cycle acids, were shown to be able to oxidize them after their cell structure had been destroyed by freezing and thawing, etc. (for instance Lynen and Lynen (36) for yeast cells, Cochrane and Peck (59) for *Streptomyces coelicolor*). This was ascribed to the removal of permeability barriers ; and the fact that some oxidation of cycle acids could be demonstrated by dead cells or in cell free extracts was taken as evidence that the tricarboxylic acid cycle was operating in these microorganisms. This argument does not seem conclusive to the reviewer.

In the first place, in most cases the oxidative activity of the treated cells or cell extracts towards the various cycle acids was found to be extremely low, not more than a few per cent of their normal rate of respiration in the intact state in the presence of glucose or acetate ; the mere presence of cycle enzymes in small amounts in the cells does not prove that the tricarboxylic acid cycle operates as a whole and is the major pathway of oxidative carbohydrate metabolism.

Furthermore, it has been demonstrated by Foulkes (60) that, while the permeability of yeast cells to citrate could be increased by freezing and thawing, the cells thus treated were still unable to metabolize this substance ; a considerable utilization of citrate was brought about, however, by treatment of the frozen and thawed cells with chloroform. Permeability barriers to citric acid are therefore not sufficient to explain the inability of the cells to metabolize this substrate.

The presence of some cycle enzymes in cell free extracts of *P. chrysogenum* has been demonstrated by different groups of workers. Casida and Knight (61) found that such extracts showed strong succinic dehydrogenase activity with oxygen as hydrogen acceptor in Warburg vessels, or with 2,6-dichlorophenolindophenol as hydrogen acceptor in Thunberg tubes. Some small degree of malic dehydrogenase, α-ketoglutaric and *iso*citric dehydrogenase activity could be demonstrated in Thunberg tubes with 2,6-dichlorophenolindophenol as hydrogen acceptor. Citrate, fumarate, malate, pyruvate and acetate were not oxidized to an appreciable degree by the extracts. The extracts were also able to bring about the condensation of acetylphosphate and oxaloacetate to citrate. On the basis of this evidence, Casida and Knight conclude that the tricarboxylic acid cycle « could be present in *P. chrysogenum* ». Olson and Chain (62) were also able to demonstrate that cell free extracts of *P. chrysogenum*, grown on acetate, are able to oxidize succinate ; furthermore, they found that in fresh extract oxidation of citrate could be observed (figure 3). The citrate oxidizing system was found to be extremely unstable and the extracts rapidly lost their activity on standing. The maximal rates of oxidation of the substrates in cell free extracts represented, however, as they did in frozen and thawed cells only, a very small fraction (less than 2 %) of the oxidative activity of the whole cells towards acetate. Olson and Chain (62) also demonstrated the occurrence of *iso*citric dehydrogenase, aconitase and fumarase in their cell free extracts of *P. chrysogenum* ; these also contained an active oxaloacetate decarboxylase.

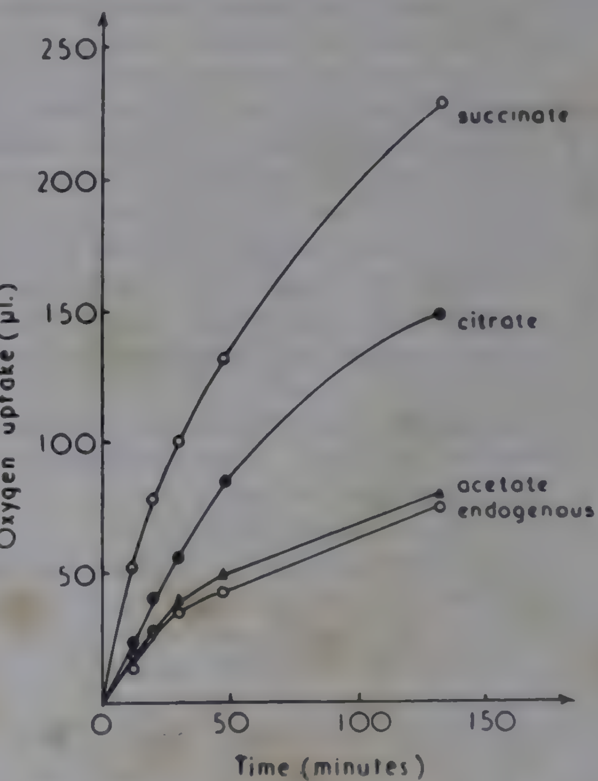



FIG. 3. — Oxidation of organic acids by cell-free extracts. Warburg vessels contained the following final concentration in 3 ml. : 0.5 ml. extract (3.8 mg. protein N), 0.017 M substrate, 0.07 M sodium phosphate, pH 6.8 ; 0.2 ml. 30 % KOH in the center well. Vessels were incubated at 25° C. for 2 hours.

While on the basis of the evidence at present available it is not possible to state with certainty that the tricarboxylic acid cycle represents the major pathway of carbohydrate and acetate oxidation in *P. chrysogenum*, there is no doubt that citrate is rapidly formed from acetate by this mould. This was shown by Koffler and his colleagues, using $^{14}$C-methyl labelled acetate. After aerobic incubation of acetate grown mycelium of *P. chrysogenum* for fifteen seconds with methyl labelled acetate and subjecting alcohol extracts to quantitative

TABLE X

*Effect of incubation time on $^{14}CH_3COOH$ incorporation into mould fractions*

| Experiment N° | 3 | 4 | 5 |
|---|---|---|---|
| Incubation time | 2 minutes | 10 minutes | 30 minutes |
| μ-moles $O_2$ consumed | — | 4.4 | 11.8 |
| μ-moles $O_2$ endogenous | — | 2.2 | 5.9 |
| μ-moles acetate added | 30 | 30 | 30 |
| Orig. c.p.m. in acetate | $2 \times 10^6$ | $2 \times 10^6$ | $2 \times 10^6$ |
| Utilized acetate (c.p.m.) | 68 000 (100 %) | 193 000 (100 %) | 556 000 (100 %) |
| Steam distilled remainder | 61 000 | 183 000 | 495 000 |
| aqueous phase | 40 000 (59 %) | 105 000 (55 %) | 295 000 (53 %) |
| ether extract | 3 000 (4 %) | 7 000 (4 %) | 20 000 (4 %) |
| mycelial residue | 1 300 (2 %) | 2 000 (1 %) | 22 000 (4 %) |
| Respiratory $CO_2$ | 6 700 (10 %) | 10 000 (5 %) | 61 000 (11 %) |
| Total recovery | 51 000 (75 %) | 124 000 (65 %) | 398 000 (72 %) |

Warburg vessels contained 2.7 ml. mould suspension (10 mg. dry weight) in 0.08 M potassium phosphate, pH 6.0, with 0.2 ml. 30 % KOH in the center well and 0.1 ml. 9 N $H_2SO_4$ in the sidearm ; 0.3 ml. $^{14}CH_3COOH$ was added at zero time, and the vessels were incubated at 25° C. At the prescribed times $H_2SO_4$ was added to stop the reaction. The KOH was removed, and the suspension was steam distilled to remove unutilized acetate. The mycelium was filtered off and the filtrate extracted with ether.

radiochromatography, they were able to show that the extracts contained labelled citrate and glutamate (44.6 and 23.2 % of the total activity of the paper strip ; the rest of the activity was due to an unidentified compound). The citrate rapidly disappeared ; after 30 seconds incubation, the percentage of radioactivity of citrate had decreased to 18.0 %, while that of glutamate had increased to 69.9 %. On incubation for thirty minutes a number of labelled compounds could be detected, among them glycolate, succinate, malate and α-ketoglutarate, and the amino acids aspartate, alanine, serine and glycine.

Olson and Chain (62) also studied extensively the fate of methyl labelled acetate. The distribution of radioactivity in the different fractions during the course of the incubation is indicated in table X.

Most of the activity remained in the aqueous layer, whereas the ether extracts showed little activity.

Chromatography of the latter showed the presence of labelled citrate, malate and succinate ; the radioactivity of the aqueous layer was found to be due to the three amino acids, glutamate, aspartate and alanine, glutamate forming the largest proportion. Stepwise degradation of the glutamate was carried out, with the results reported in table XI.

The greatest part of the activity was in carbon 4, indicating that condensation of the acetate with a four-carbon compound had taken place. The presence of radioactivity to about an equal degree in carbons 2 and 3 indicates that randomization of the four-carbon compound had taken place, probably through equilibration with fumarate.

The almost complete absence of radioactivity in the carboxyl carbons shows that carboxylation had not taken place to an appreciable degree under the conditions of the experiments, in which about 11 % of the activity of the acetate utilized appeared as carbon dioxide.

While it is clear from the experiments of Koffler *et al.* and Olson and Chain that *P. chrysogenum* readily performs the condensation of acetate to citrate, it is by no means established by these experiments that the citric acid cycle is the major pathway of glucose and acetate oxidation. Freezing and thawing of the mycelium completely abolishes its ability to oxidize acetate ; however, incorporation of methyl labelled acetate still takes place to about the same degree as with the intact mould and the distribution of radioactivity was also found to be very similar. Some incorporation of radioactive acetate also occurs in cell free extracts.

TABLE XI

*Incorporation of $^{14}CH_3COOH$ into metabolic glutamic acid*

| Carbon Atom | c.p.m. | % Total activity |
|---|---|---|
| Glutamic acid (I) | 43.9 | |
| (complete combustion) (II) | 45.6 | |
| Average | 44.7 | 100 |
| Carboxyl C-1 | 12.1 | 5 |
| Amino C-2 | 40.0 | 18 |
| Methylene C-3 | 36.1 | 16 |
| Methylene C-4 | 124.3 | 56 |
| Carboxyl C-5 | 2.0 | 1 |
| Total recovery | | 96 |

The metabolic glutamic acid was isolated from the aqueous phases of experiment 4 and 5 by chromatography in ammoniacal phenol-water (4 : 1), elution with water, and crystallization as the hydrochloride after the addition of 200 mg. carrier glutamate. After recrystallization the specific activity of the crystals and of the filtrate was identical. The complete combustion of glutamic acid, and its stepwise degradation were carried out as described in the experimental section. Each $BaCO_3$ sample was ground in a glass mortar in $H_2O$, plated in a layer of 'infinite thickness', and counted to 2000 events (*ca.* 40 min.) over background (error = 5 %).

TABLE XII

*The effect of freezing thawing on the ability of mould suspensions to assimilate $^{14}CH_3COOH$*

| Experiment N° | 6 | 7 | 8 | 9 |
|---|---|---|---|---|
| Mould treatment | Fresh | Fresh | Frozen | Frozen |
| Incubation time | 20 minutes | 20 minutes | 45 minutes | 45 minutes |
| $\mu$-moles Na acetate | 30 $\mu$-moles | 30 $\mu$-moles | 30 $\mu$-moles | 30 $\mu$-moles |
| Orig. c.p.m. in acetate | 650 000 | 650 000 | 650 000 | 650 000 |
| Utilized acetate | 125 000 (100 %) | 130 000 (100 %) | 110 000 (100 %) | 70 000 (100 %) |
| Steam distilled residue | 88 000 | 96 000 | 80 000 | 49 000 |
| ether extract | 11 000 (9 %) | 13 000 (10 %) | 11 000 (10 %) | 10 000 (14 %) |
| aqueous phase | 66 000 (53 %) | 86 000 (66 %) | 66 000 (60 %) | 26 000 (37 %) |
| amino acids | 31 000 | 26 000 | 14 000 | 8 400 |
| uncharged compounds | 11 000 | 15 000 | 11 000 | 13 000 |
| Mycelium | 30 000 (24 %) | 25 000 (19 %) | 18 000 (16 %) | 15 000 (21 %) |
| extract | 6 000 | 16 000 | 15 000 | 10 000 |
| residue | 9 000 | 9 000 | 9 000 | 5 000 |
| $BaCO_3$ | 7 000 (6 %) | 9 000 (7 %) | 12 000 (11 %) | 8 000 (11 %) |
| Gross recovery | 114 000 (92 %) | 133 000 (102 %) | 107 000 (97 %) | 59 000 (84 %) |

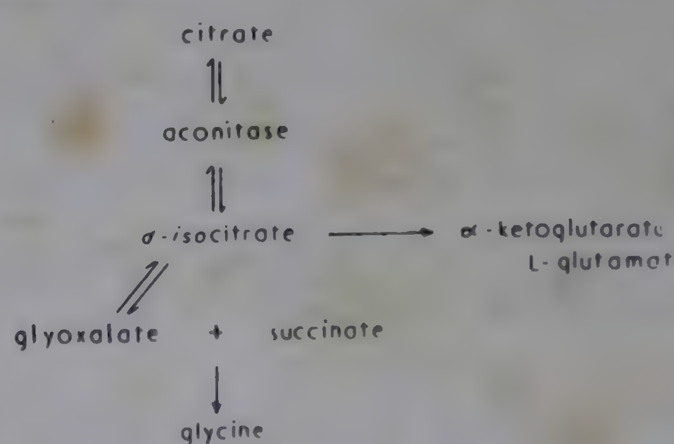
Warburg vessels contained 2.7 ml. of a thick mould suspension (*ca.* 100 mg. dry weight), 0.3 ml. $^{14}CH_3COOH$ (30 $\mu$-moles) in the sidearm, and 0.2 ml. 30 % KOH in the centerwell. The mould suspensions for experiments 8 and 9 were frozen in dry ice-acetone prior to incubation. After equilibration at 25° C., substrate was added and the vessels were incubated as indicated above. The reaction was stopped with 0.2 ml. 9 N $H_2SO_4$ added through the sidearm. The suspensions were treated as outlined in the experimental section.

In the intact mould the ethereal extracts of the frozen mould contained radioactive citrate, malate and succinate; in the aqueous extracts glutamic acid was the main radioactive compound. About 40 % of the total activity of methyl labelled acetate utilized was recovered in glutamate. In view of the fact that the above-mentioned products were formed by a mycelium which did not respire, their mere occurrence is no proof that they have been formed by oxidative mechanisms. The succinate may have originated from the action of *iso*citric lyase on citrate (see below).

Further evidence for citrate formation by *P. chrysogenum* was provided by Hockenhull, Wilkin and Winder (63) and Hockenhull, Herbert, Walker, Wilkin and Winder (55), who isolated α-ketoglutaric acid from culture filtrates of shake flask cultures of *P. chrysogenum* after incubation with various acids such as lactate, succinate, fumarate, and malate in the presence of arsenite. No α-ketoglutarate was isolated during the oxidation of acetate in the presence of arsenite, but pyruvate could be detected under these conditions.

### *Cleavage of citric acid in cell free extracts of* P. chrysogenum. iso-*Citric lyase*

Citric acid disappears rapidly when added to arsenite-containing cell free extracts of *P. chrysogenum* (62). Among the breakdown products glyoxalate, succinate, glutamate, α-ketoglutarate and glycin were detected. Further analysis of this reaction (37) showed that citrate was cleaved enzymatically by the extracts in two different ways, depending on the conditions. Fresh extracts brought about complete cleavage into glutamate and α-ketoglutarate in the presence of arsenite, magnesium ions and a trace amount of triphosphopyridine nucleotide (diphosphopyridine nucleotide was inactive). Extracts aged for 4 hours at 0° C., or dialysed, produced a quantitative cleavage of citrate into glyoxalate and succinate in the presence of semicarbazide; in the presence of arsenite less glyoxalate was formed, but a large amount of glycine accumulated. The enzyme system responsible for the cleavage of citrate into succinate and glyoxalate has been termed an *iso*citric lyase system, because the immediate precursor of the cleavage products is almost certainly *iso*citric acid. The two pathways of cleavage of citrate by cell free extracts of *P. chrysogenum* may be represented as follows :




The cleavage of citrate into succinate and glyoxalate is anaerobic and reversible. A similar enzyme system was isolated from bacteria by Campbell, Smith and Eagler (64), Smith and Gunsalus (65).

*iso*Citric lyase has also been found in other fungi, for instance *Aspergillus niger* and *Rhizopus* sp. This enzyme may account for the formation of glyoxylic acid, which has been known for many years as a mould metabolite, but the origin of which was obscure. It certainly provides a mechanism for the formation of glycin from acetate, which has been shown to occur in the mycelium

of *P. chrysogenum*; the formation of glyoxalate from acetate by *A. niger* was demonstrated many years ago by Challenger, Subramanian and Walker (66). Its function, if any, in the formation of oxalate (see above) remains to be elucidated.

### *Acid formation in* Rhizopus

Members of the genus *Rhizopus* convert glucose into lactic acid (for literature up to 1948 see 67). Anaerobically one mole of lactate, one mole of ethanol, and one mole of carbon dioxide are formed from one mole of glucose. Aerobically the yield of lactate may be higher, up to 1.6 mols per mole of glucose, while the alcohol production is greatly reduced. Isotope studies on the mechanism of aerobic glycolysis by *Rhizopus* have been made by Carson, Foster, Jefferson, Phares and Anthony (68) and Gibbs and Gastel (69). Using methyl-$^{14}C$ labelled pyruvate as tracer, Carson *et al.* (68) found that the lactate formed aerobically from glucose by *Rhizopus* contained 94-95 % of the radioactivity in the methyl carbon, whereas using carbonyl-$^{14}C$ labelled pyruvate as tracer 87 % of the activity was found in the α-carbon. This is in accordance with the theory that the major pathway of aerobic lactate formation by *Rhizopus* proceeds *via* the Meyerhof-Embden glycolytic pathway.

Similar conclusions were drawn by Gibbs and Gastel (69). These authors used glucose 1-$^{14}C$ and glucose-3,4-$^{14}C_2$ as tracers, and examined the distribution of the radioactivity in the carbon dioxide and the individual carbon atoms of the lactate and ethanol formed under anaerobic and aerobic conditions. According to the Meyerhof-Embden glycolytic scheme 1-$^{14}C$-labelled glucose should lead to methyl labelled lactate, while 3,4-$^{14}C_2$-labelled glucose should lead to carboxyl labelled lactate; the ethanol formed from 1-$^{14}C$-labelled glucose should contain all its activity in its methyl groups, while that formed from 3,4-$^{14}C_2$-labelled glucose should be devoid of activity; finally, the carbon dioxide formed from 1-$^{14}C$-labelled glucose should be inactive, and that formed from 3,4-$^{14}C_2$-labelled glucose active. Their results, reported in tables XIII and XIV, are in complete agreement with the Meyerhof-Embden scheme of glycolysis.

The small amount of radioactivity in the carboxyl carbon of lactate with $C_1$-labelled glucose as tracer is presumably due to carbon dioxide fixation.

TABLE XIII

*Distribution of radioactivity in ethanol and lactate carbons formed anaerobically from labelled glucose by* Rhizopus orizae

| Tracer | $CO_2$ | Ethanol | | Lactate | | |
|---|---|---|---|---|---|---|
| | | $CH_3$ | $CH_2OH$ | COOH | CHOH | $CH_3$ |
| Glucose-1-$^{14}C$ | — | — | — | 0.5 | 0.5 | 16.0 |
| Glucose-1-$^{14}C$ | 0.5 | 14.1 | 0.9 | 0.2 | 0.2 | 3.8 |
| Glucose-3,4-$^{14}C_2$ | 13.0 | 0 | 0 | 13.2 | 0.35 | 0.5 |
| Glucose-3,4-$^{14}C_2$ | — | — | — | 14 | 0.2 | 0.2 |

After Gibbs and Gastel (69).

TABLE XIV

*Distribution of radioactivity in lactate formed aerobically from $^{14}C_1$-labelled glucose*

| Medium | COOH | CHOH | $CH_3$ |
|---|---|---|---|
| Phosphate . . . . . . . . . . | 0.3 | 0.1 | 3.4 |
| Phosphate . . . . . . . . . . | 0.3 | 0.1 | 2.2 |
| Calcium carbonate. . . . . . | 0.2 | 0.3 | 5.9 |
| Calcium carbonate. . . . . . | 0.7 | 0.6 | 10.5 |

After Gibbs and Gastel (69).

Carson *et al.* (68) obtained evidence that in addition to the glycolytic pathway there exists, if to a minor degree, an oxidative pathway of lactic acid formation by *Rhizopus via* the dicarboxylic acids. Using labelled fumaric acid as tracer (formed by *Rhizopus* from methyl labelled ethanol, see below; the ratio of the specific activities of the CH and the COOH carbon was 4.2 : 1), they found that the lactic acid produced aerobically from non-radioactive glucose was radioactive, and that the specific activity was distributed evenly between the $CH_3$ and the CHOH, while the carboxyl group contained about a quarter of the specific activity of the $CH_3$ and CHOH groups. Furthermore, these authors found that, when methyl labelled alcohol was used as a tracer, the lactate formed from non-radioactive glucose was radioactive, with a specific activity of 307, 295 and 106 c.p.m. m-mole C for the methyl, carbinol and carboxyl groups respectively, indicating that both the methyl and carbinol groups of the lactate derived from the methyl groups of ethanol. One possible interpretation of this result is that intermediate formation of a four-carbon compound by '$C_2 + C_2$' condensation had taken place, and that this compound was subsequently decarboxylated.

When alcohol was used as the sole substrate, the authors found that the *Rhizopus* strain produced mainly fumaric acid, instead of lactate. Using methyl labelled ethanol as a tracer, the authors isolated and stepwise degraded the malate in equilibrium with the fumarate which appeared in small amounts in the culture fluid under these conditions, and found that 77 % of the activity was equally distributed between the two non-carboxyl carbons; the rest was in the two carboxyl groups, also equally distributed.

From these results they concluded that ethanol was converted to lactate oxidatively *via* the formation of dicarboxylic acids, and that these were formed *via* a '$C_2 + C_2$' condensation.

This type of condensation was suggested without experimental evidence as a mechanism for the formation of dicarboxylic acids many years ago by Thunberg (70) and Knoop (71), and is often referred to as the Thunberg-Knoop dicarboxylic acid cycle. The evidence of Carson *et al.* with the lactic acid producing strain of *Rhizopus* appeared to provide conclusive experimental evidence for this scheme. Similar evidence was obtained earlier by Foster, Carson, Anthony, Davis, Jefferson and Long (72) and Foster and Carson (73), using a strain of *Rhizopus nigricans* which produces fumaric acid from alcohol as its main metabolic product. The yields of

fumarate were so high (50 to 80 %) that it could not have originated from the sequence of the tricarboxylic acid cycle reactions. Using methyl labelled ethanol as a tracer, they found that the methine carbons of the fumaric acid formed contained the same specific radioactivity as the methyl groups of the ethanol. The carboxyl groups were also radioactive, containing about one-fifth the activity of the methine groups, so that the total radioactivity of the fumarate was higher than that of two moles of methyl labelled ethanol. Radioactivity must have been incorporated into the carboxyl groups of fumarate, probably through carboxylation of a three-carbon compound. When carbinol labelled ethanol was used as tracer, all the radioactivity of the fumarate formed resided in the carboxyl groups, while the methine carbons were free from radioactivity. This is again strong evidence for a mechanism of fumarate synthesis involving direct '$C_2 + C_2$' condensation.

However, Jefferson, Foster, Phares and Carson (74) and Jefferson and Foster (75) found that addition of radioactive formate as tracer to non-radioactive glucose resulted in the formation of radioactive metabolic end products, both in aerobic shake cultures of *Rhizopus* strains which convert glucose predominantly into ethanol and lactate together with small amounts of fumarate, and in aerobic shake cultures of other *Rhizopus* strains which produce predominantly ethanol and fumarate. Stepwise degradation of the radioactive lactate, fumarate, and ethanol formed under these conditions gave the following results (table XV).

TABLE XV

*Distribution of radioactivity in principal metabolic products formed aerobically from glucose by* Rhizopus, *in presence of $H^{14}COOH$ as tracer*

| Metabolite formed | Strain predominantly producing lactate (c.p.m./μ-mol.C) × $10^{-2}$ | Strain predominantly producing fumarate (c.p.m./μ-mol.C) × $10^{-2}$ |
|---|---|---|
| Lactate : | | |
| $CH_3$— | 826 | |
| —CHOH— | 18.2 | |
| —COOH | 22.7 | |
| Fumarate : | | |
| —CH=CH— | 379 | 57.9 |
| —COOH | 111 | 94.2 |
| Alcohol : | | |
| $CH_3$— | 38.0 | 5.5 |
| —$CH_2$OH | 7.3 | 16 |

After Jefferson, Foster, Phares and Carson (74).

These results demonstrate that formate is a precursor of the methyl groups of the lactate and ethanol and of the methine groups of the fumarate formed from glucose. With the strain making chiefly lactic acid, the bulk of the radioactivity resided in the methyl carbon of the lactate and ethanol; with the strain predominantly forming fumarate, the carboxyl of this acid contained more radioactivity than the methine carbons. Radioactive formate was also found to be fixed by the lactate-producing strain of *Rhizopus* under anaerobic conditions (75); both the lactate and alcohol formed were radioactive, and the bulk of the activity resided in the methyl groups (table XVI).

TABLE XVI

*Distribution of radioactivity in lactate and alcohol formed aerobically and anaerobically from glucose in presence of $H^{14}COOH$ by* Rhizopus

| Metabolite | Aerobic | Anaerobic |
|---|---|---|
| Lactate : | | |
| $CH_3$— . . . . . . . . . | 151 | 117 |
| —CHOH— . . . . . . | 8.5 | 1 |
| —COOH . . . . . . . | 10.5 | 2.2 |
| Ethanol : | | |
| $CH_3$— . . . . . . . . . | 22.3 | 7.5 |
| —$CH_2$OH . . . . . . . | 1.6 | 0.1 |

After Jefferson and Foster (75).

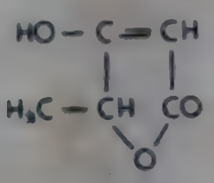
The respiratory carbon dioxide formed was strongly radioactive; it is likely that the radioactivity in the carboxyl groups of the fumarate and lactate which appears in the aerobic experiments is due to carbon dioxide fixation and oxidative recycling *via* a di or tricarboxylic acid cycle. The fixation of carbon dioxide under aerobic conditions is greatly reduced; the main reaction which formate undergoes under these conditions is fixation as methyl. The mechanism of this formate transformation in methyl groups is not yet understood. The fact that it occurs, and apparently to an appreciable extent, has, however, important implications for the interpretation of the experiments reported above on the incorporation of the radioactivity from methyl labelled ethanol in the methine groups of fumarate. While the presence of radioactivity in the two neighbouring carbon atoms of the fumarate molecule can be explained by a direct '$C_2 + C_2$' condensation, an alternative equally possible explanation is that part of the ethanol is oxidized to formate, which in turn is condensed with a radioactive two-carbon compound derived from ethanol in such a way that the resulting three-carbon acid is α,β-labelled. That formate is formed from alcohol during fumaric acid production from ethanol by *Rhizopus* has been proved by Jefferson and Foster (75), who isolated radioactive formate after addition of methyl labelled ethanol.

When the glucose conversion to fumarate by *Rhizopus* was allowed to proceed in the presence of arsenite and $H^{14}COOH$, radioactive pyruvate could be isolated; the ratio of counts in the methyl, carbonyl, and carboxyl carbons was 43 : 4.3 : 1.

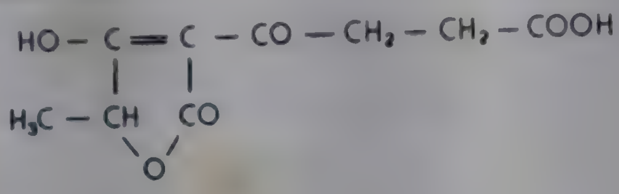
The precursor of pyruvate formed by fixation of formate is as yet unknown; in propionic acid bacteria formate is known to be incorporated in the α- and β-carbons of propionic acid, and in rat liver into the β-carbon of serine.

*Observations of the formation of tetronic acids from glucose by* Penicillium Charlesii *G. Smith*

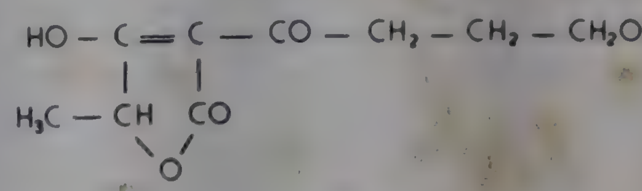
Raistrick and his collaborators have shown that the mould *Penicillium charlesii* G. Smith transforms glucose in a series of tetronic acids (for review see 1). The structures of these are listed below :



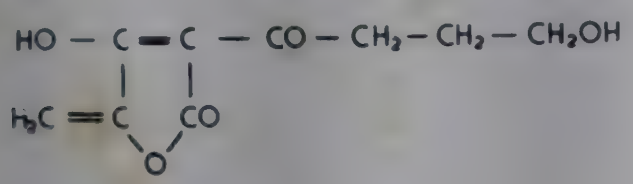
γ-methyl tetronic acid



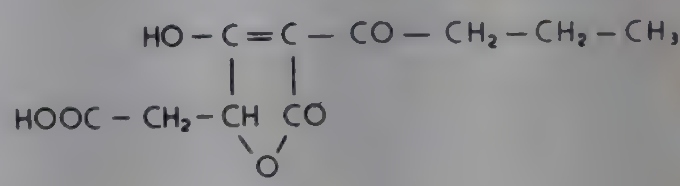
carolinic acid



carolic acid



dehydrocarolic acid



carlosic acid

Very little is as yet known about the mechanism of their biosynthesis, but Simonart and DeKeyser (76), using the replacement method with surface cultures, have shown that, when the replacement solution contained pure glucose, the $C_9$ compound carolic acid was the main metabolic reaction product, while on Czapek-Dox or Raulin-Thom medium a mixture of the above-mentioned tetronic acids was formed. In the presence of calcium carbonate in the glucose-containing replacement solution, citric acid could be isolated in addition. It could be demonstrated that citric acid was transformed into carolic acid, and the authors consider on the basis of this fact that citrate is a precursor of carolic acid in the normal metabolism of the mould.

Tracer studies will undoubtedly provide valuable new evidence on the mechanism of biosynthesis of the tetronic acids.

*Glucose transformation by* Penicillium brevi-compactum

The studies of Raistrick and his collaborators (for review see 1), have shown that this mould converts glucose into a series of phenolic substances, among which mycophenolic acid, 3,5-dihydroxy, 2-carboxybenzoylmethylketone, 3,5-dihydroxy, 2-carboxylphenacetylcarbinol 3,5-dihydroxy-2-carboxybenzoylmethylketone hydrate and 3,5-dihydroxyphthalic acid have been identified. Godin (77) has tried by means of chromatographic methods to identify intermediates of glucose metabolism appearing in the culture fluid of this organism under conditions of surface and submerged culture in Czapek-Dox medium. After about 4 weeks, surface cultures were shown to contain the above-mentioned phenolic substances, some in very small quantities. In addition, gluconic acid was found in appreciable amounts, and the presence of very small quantities of glucuronic, 2-ketogluconic, malic and citric acids could also be established. Two pentoses, arabinose and ribose, were identified, and their presence in conjunction with the appearance of considerable quantities of gluconic acid suggests that glucose may be oxidized by the direct hexosephosphate shunt.

The presence of 2-ketogluconic acid, ribose, and glucuronic acid in culture filtrates of *P. brevi-compactum* had previously been established by Simonart and Godin (78) ; the formation of glucuronic acid is interesting, since it is the first time that this substance has been found as a metabolic mould product.

Further evidence in favour of the pathway of glucose oxidation by *P. brevi-compactum via* gluconic acid and pentose formation was obtained by Godin (79) by studies of the inhibition of glucose and gluconic acid utilization by iodoacetate, sodium fluoride, cupric chloride and sodium azide. The appearance of fructose and glycerol among the metabolic products formed in the presence of cupric ions indicates that a glycolytic pathway along the Meyerhof-Embden scheme exists.

In submerged culture, where the oxidative metabolism of glucose is more intense, no phenolic substances except very small quantities of 3,5-dihydroxy-2-carboxybenzoylmethylketone are produced, but considerable amounts of gluconic and citric acids were formed, and several other acids, such as succinic, malic, fumaric and ketoglutaric acids, were identified ; the latter were present in small quantities only.

With a glucose-containing replacement medium, put under surface cultures, a difference in glucose metabolism was noted depending on whether calcium carbonate was present or not.

In the presence of calcium carbonate malic, succinic, fumaric and oxaloacetic acids were identified, and considerable quantities of dihydroxy acetone accumulated ; in the absence of calcium carbonate only a very small quantity of dihydroxyacetone was produced and no cycle acids could be detected.

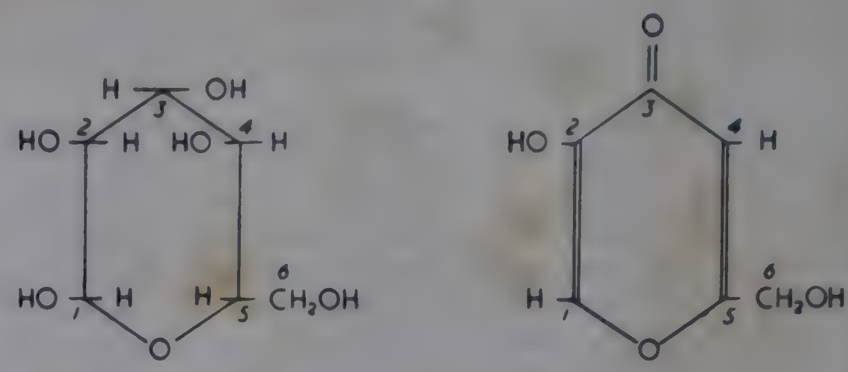
Larger amounts of phenolic substances were produced in the presence of calcium carbonate than in its absence. The effect of calcium carbonate on the course of the metabolism of *P. brevi-compactum* was not just a pH effect, for it could not be reproduced by substituting phosphate for calcium carbonate. The reviewer believes that calcium carbonate displaces the equilibrium, existing between the production of phenolic substances and

glucose oxidation *via* the cycle acids, in favour of the latter. Isotope experiments will be required to check this hypothesis.

In addition to the phenolic substances discovered by Raistrick and his collaborators, Godin (80) detected by means of chromatographic methods the presence of six other phenolic substances in the culture fluid of *P. brevi-compactum*. Using replacement media and surface growth, Godin (81) found that the phenolic substances were formed from a variety of carbon sources, in addition to glucose, including fructose, arabinose, D-ribose, and glycerine, and citric, succinic, and malic acids. *Iso*Citric acid, in contradistinction to citric acid, was not found to be metabolized by preformed mycelium of *P. brevi-compactum*. On the basis of this finding, as well as of transformation studies in replacement media of different di and tricarboxylic acids, Godin (82) comes to the conclusion that the tricarboxylic acid cycle does not operate in its entirety in *P. brevi-compactum* and is in favour of metabolic pathways involving a Thunberg-Knoop '$C_2 + C_2$' condensation according to a scheme proposed many years ago by Chrzaszcz and Tiukow (83). In this case again, really conclusive evidence will be obtained only by application of the isotope tracer technique.

### *Mechanism of kojic acid synthesis by* Aspergillus

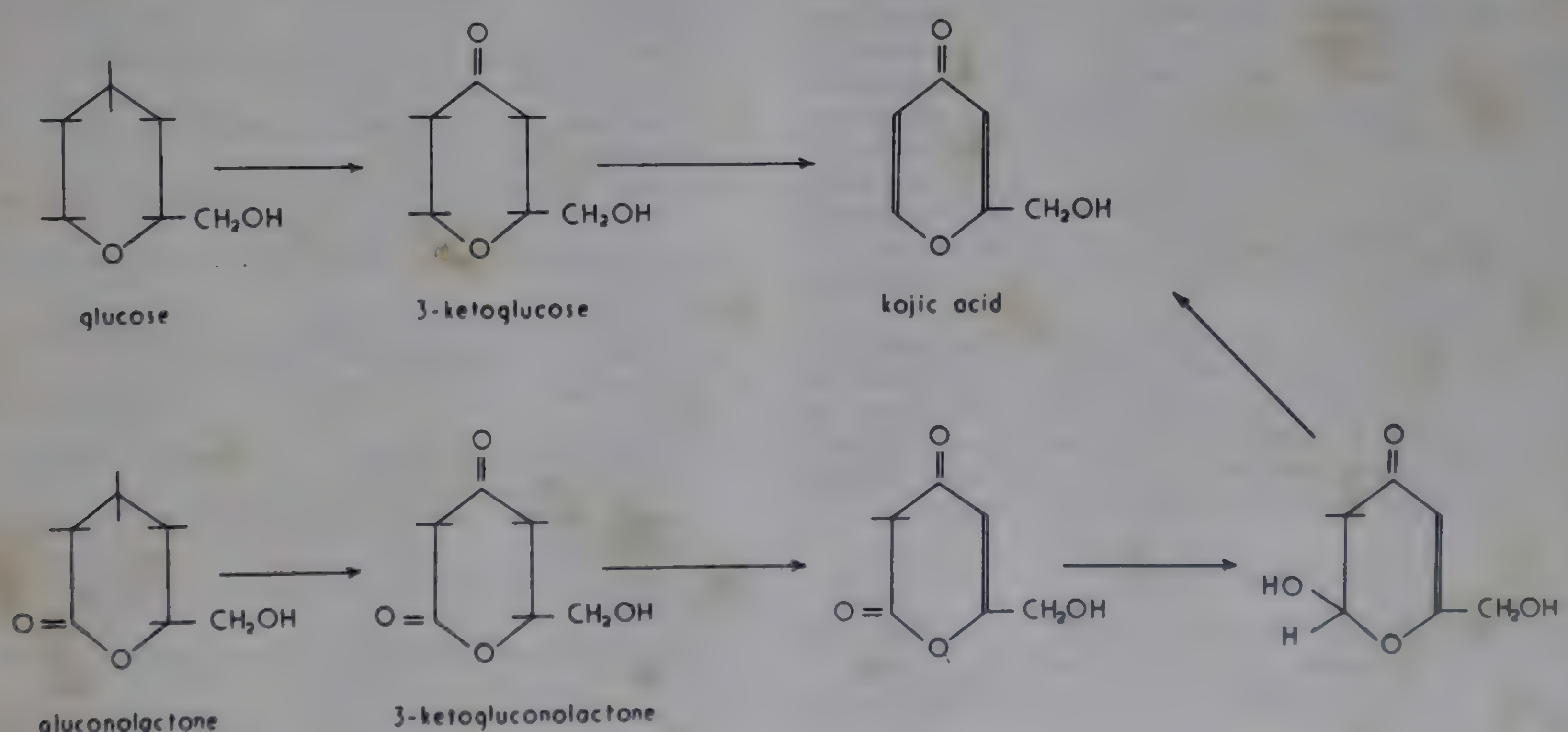
Kojic acid is a pyrone derivative produced from glucose and a variety of other carbon sources by several species of *Aspergillus*, such as *A. flavus*, *A. oryzae*, and others. On the basis of metabolic studies, several mechanisms for its formation have been proposed (for earlier literature, see Walker, 11, and Foster, 67), but without conclusive experimental evidence. The problem of the mechanism of the biosynthesis of kojic acid has been reinvestigated by Arnstein and Bentley (84, 85, 86), with $^{14}C$-labelled tracer compounds; this approach has given clearcut and unequivocal results. 1-$^{14}C$-glucose and 3,4-$^{14}C$-glucose were used as tracers for the study of the conversion of glucose to kojic acid, and new methods of stepwise degradation of kojic acid were developed, by means of which the radioactivity of each of its carbon atoms could be determined (for numbering see below).



glucose ($C_6H_{12}O_6$) kojic acid ($C_6H_6O_4$)

With 1-$^{14}C$-glucose as tracer, the bulk of the radioactivity (68 to 87 %) was recovered in carbon 1 of the kojic acid molecule; with 3,4-$^{14}C$-glucose as tracer, the radioactivity was located mainly in 3-C and 4-C. These results demonstrate clearly that the main pathway of kojic acid formation from glucose is the direct conversion of the latter, without cleavage of the carbon atoms, into three-carbon or smaller-carbon fragments. Several possible mechanisms for the conversion of glucose into kojic acid can be conceived. One of these, suggested by earlier workers, involves the formation of 3-keto-glucose as intermediate, followed or preceded by dehydration.

The other involves the intermediate formation of gluconic acid and 3-ketogluconic acid. The latter may then be reduced to 3-ketoglucose and converted to kojic acid by dehydration, as above, or it may first be dehydrated and the unsaturated lactone reduced and converted to kojic acid by a second dehydration. These pathways are indicated in the following scheme :



Support for the pathway *via* gluconic acid is seen in the fact that glucose oxidases bringing about the oxidation of glucose to gluconic acid are frequently encountered in moulds, and ketogluconic acids have been established as mould metabolites by several investigators.

In addition to the direct conversion of glucose to kojic acid, there exist minor pathways involving the cleavage of the glucose carbon chain.

One of these is the cleavage in triose units. When 1-C-labelled glucose was used as tracer, part of the radioactivity (6 to 16 %) was found in carbon 6 of kojic acid. Furthermore, the glucose itself, when isolated at the end of the experiment, contained some radioactivity in carbon 6. This strongly suggests that the glucose is, at least to some extent, in equilibrium with trioses. Arnstein and Bentley found, in fact, aldolase activity in the mycelium of their strains of *A. flavus-oryzae*, as did Jagannathan and Singh (31, 32) (see above), in *A. niger*, and there is strong evidence from isotope experiments that glycolytic cleavage of glucose according to the Embden-Meyerhof scheme does occur in *Aspergillus* (see above under citric acid formation). Kojic acid can be synthesized by *A. oryzae* from dihydroxyacetone as sole carbon source; with 2-$^{14}$C-dihydroxyacetone as tracer, about 60 % of the total radioactivity in the kojic acid formed was recovered equally distributed between 2-C and 5-C, suggesting that a symmetrical condensation had taken place. The rest of the radioactivity was about equally divided between 1-C, 3-C, and 6-C with a small amount present in 4-C. In the presence of glucose, the bulk of the radioactivity of 2-$^{14}$C-dihydroxyacetone added as tracer was recovered in 5-C, possibly due to preferential condensation of the radioactive triose with non-radioactive triose from glucose.

A study of the incorporation into kojic acid of various radioactive two- and three-carbon tracers, with non-radioactive glucose as the main substrate, such as carboxyl labelled acetate, methyl labelled acetate, carboxyl labelled glycine, methyl labelled glycine, methyl labelled pyruvate, and carboxyl labelled acetone, showed that only a small amount of the radioactivity of these compounds, in most cases less than 1 %, was recovered in the kojic acid formed. This excluded their participation in kojic acid formation as a major pathway. All carbon atoms of the kojic acid molecule had some radioactivity, but the sum of the activity of 4-C, 5-C and 6-C was much greater than that of 1-C, 2-C and 3-C. This suggested, as in the case of dihydroxyacetone, a condensation of a radioactive three-carbon compound with a non-radioactive triose unit.

The similarity of the distribution of the radioactivity in the kojic acid molecule deriving from the two-carbon compounds and pyruvate suggests that they may be transformed into a common triose precursor of kojic acid. Formate and carbon dioxide were not utilized to an appreciable degree for kojic acid synthesis.

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*Imprimé en Belgique*

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